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Total lipids and phospholipids of turkey tissues

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TOTAL LIPIDS AND PHOSPHOLIPIDS OF TURKEY TISSUES

by

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INTRODUCTION

The distribution of lipids in animal tissues is widely variable in both quantity and type. Although the total lipid content of a given tissue may vary within wide limits, this variation is generally due to changes in the glyceride or fat content. The lipid fraction composed of cholesterol, phospholipids and glycolipids is relatively more constant in amount. The sum of cholesterol, phospholipids and glycolipids in a tissue is commonly referred to as "essential" lipid. These lipids are considered essential components of protoplasmic structures, while on the other hand, the fat content of tissues is a reserve food material and may vary from a relatively large amount during good nutrition to practically nothing during starvation. Phospholipids constitute the bulk of essential lipids in tissues as well as in organs.

The ubiquitous occurrence of phosphatides as components of living organisms has led to the implication that these compounds have a variety of functions: 1) concerned with the structure of certain cellular components and with metabolic processes related to these structures; 2) involved in other important functions of the cell such as protein synthesis and secretion; 3) involved in the absorption and transport of fat and certain of the phosphatides may be

required for normal blood coagulation; 4) principal lipid components of myelin sheath of nerve; and 5) phospholipid is required for maximal activity in at least three segments of the electron-transfer chain.

Published work is meager on the analysis of individual phospholipids of organs and tissues of various species of animals. No data were found on the phospholipid content of turkey tissues.

Several workers have demonstrated that oxidative rancidity is responsible for off-odors and flavors which develop during the storage of meat. The lipid fractions primarily involved in the oxidative reactions are proteolipids and phospholipids rather than triglycerides. Rancidity in meat results from the oxidative decomposition of unsaturated fatty acids. The concentration of unsaturated fatty acids in both meats and fish is highest in the phospholipid and proteolipid fractions, which are among the more labile components of foods. Their role in the deterioration of a few commodities such as eggs and milk has been studied. Little is known, however, about their contributions to spoilage mechanisms in muscle tissue.

The work reported herein was designed, therefore, to determine qualitatively and quantitatively the total lipid and major phospholipids in various turkey tissues; to investigate the effect of different storage periods on the total lipids

and phospholipid contents, and to compare the rate of oxidation of total lipid and of various phospholipid fractions from turkey muscles subjected to different methods of storage and preparation.

REVIEW OF LITERATURE

Phospholipid composition of tissues

Bloor and Snider (1934) and Bloor (1936) showed that there may be rather wide variations in the so-called "essential" or constant fraction of tissue lipids. These workers studied the cholesterol and phospholipid contents of muscles of many different animal species and concluded that muscles as a group have no definite phospholipid and cholesterol contents but that there are: 1) differences in content between heart, smooth and voluntary muscles; 2) differences in muscles of the same type in an animal depending upon the use of the muscle; and 3) differences in the same muscle in animals of different species which may vary with muscle use.

Dawson (1957) reviewed studies on the phospholipid composition of rat tissues. Lecithin and cephalin predominated in skeletal muscle while phosphatidylserine and plasmalogen occurred in smaller amounts. Christl (1953) demonstrated the presence of plasmalogen in bovine muscle.

Studies on the distribution and structure of the phospholipids in certain mammalian tissues and subcellular components have been reported previously by Gray and Macfarlane (1958), Gray (1960a,b), Macfarlane *et al.* (1960) and Macfarlane (1961). In the recent work of Gray and

Macfarlane (1961), they studied the composition of tissue phospholipid on pigeon, trout, pig and rabbit muscles. Their analyses for various tissues indicated little difference between these species in the nature of the phospholipids in the same kind of tissue, except for the virtual absence of choline plasmalogen from trout muscle. They reported that the lipid content is high in pigeon-breast muscle. About 45 percent of the total is phospholipid, 2 percent cholesterol and the remainder almost entirely triglycerides. In the trout muscle, the total lipid is much higher than that reported for cod and haddock flesh (Garcia et al., 1956), mainly owing to an increase in triglyceride content.

Detailed studies have been made on the phospholipids of the flesh of cod and haddock (Lovern, 1956a). The component phospholipids of this tissue show a general similarity in the two species. A complex mixture of phospholipids is present, in which the lecithins predominate (50 to 60 percent of the total phospholipids). Phosphatidylethanolamine is a minor component, and there are traces of plasmalogens. It is doubtful if sphingomyelin is present, and phosphatidylserine is absent.

The distribution of lecithin, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, and plasmalogen has been

measured in six tissues of the sheep (Dawson, 1960). Complete analyses were presented for the phospholipid distribution in human erythrocyte, stroma and ox heart, liver and brain (Dawson et al., 1962) in which lecithin is the predominant phospholipid followed by phosphatidylethanolamine. Phosphatidylserine, plasmalogen and sphingomyelin are also present in considerable quantity.

The lipids extracted from beef and pork muscle were fractionated into triglycerides, cephalins and a mixture of lecithins and sphingomyelin (Hornstein et al., 1961). The phospholipid content of beef is 0.8 to 1.0 percent and 0.7 to 0.9 percent in pork. The approximate composition of the phospholipid fraction for both beef and pork is cephalin, 40 to 45 percent, lecithin, 40 to 45 percent and sphingomyelin, 10 to 15 percent.

Marinetti et al. (1957) demonstrated the quantitative analysis of pig heart phosphatides. Lecithin and phosphatidylethanolamine are the major phosphatides and sphingomyelin, phosphatidylserine, inositide, phosphatide and unidentified lipids also occurred in appreciable amounts. The lipid composition of beef liver was mostly lecithin, phosphatidylethanolamine and sphingomyelin and traces of phosphatidylserine, phosphatidylinositol and several other minor uncharacterized lipids (Rouser et al., 1963).

Lecithin and phosphatidylethanolamine were found in greatest quantity in hog meat (Kuchmack and Dugan, 1963). These represented about 61 percent and 31 percent, respectively, of the total phospholipids. The remainder was distributed between phosphatidylserine and sphingomyelin at 5 percent and 3 percent, respectively. The phospholipid of hog meat was found to be slightly over 0.5 percent. They also noted variations in content of a phospholipid type in the carcass locations; belly, ham, loin and rib. The variation, although small, shows that phosphatidylethanolamine tends to be present in greater quantities in the tissues with less total lipid content while the sphingomyelin is relatively somewhat more abundant in the tissues with greater total lipid content. The lecithin content agrees quite well with the values reported by Hornstein et al. (1961) for aged pork muscle, but the cephalin content found in this study is substantially lower than reported.

The normal range of phospholipids in the liver is 1.75 to 2.48 percent in normal adult rats (Harris and Gambal, 1963).

Changes during processing and storing

Tims and Watts (1958) reported that oxidative rancidity is responsible for off-odors and flavors which develop during storage in muscle tissues subjected to

thermal treatment. Younathan and Watts (1959) observed that these organoleptic changes can be correlated with rancidity when determinations are made on the whole tissue, but not with fat alone as extracted with the usual solvents (Watts and Peng, 1947 and Watts et al., 1948). It appears that bound lipids are contributing markedly to the reactions responsible for rancid odors and flavors in cooked meats. Younathan and Watts (1960) have recently suggested that the phospholipids play a major role in accelerating flavor deterioration in cooked meats.

Lea (1957) claimed that protein-bound phospholipids are known to be important food constituents involved in the deteriorative reactions which take place during processing and storing. These substances are among the more labile components of foods. He further studied their role in the deterioration of a few commodities such as eggs and milk.

Rancidity in meat results from the oxidative decomposition of unsaturated fatty acids. This process may be catalyzed by the ferric heme pigments of uncured cooked meat (Younathan and Watts, 1959). The concentration of unsaturated fatty acids in both meats and fish is highest in the phospholipid and proteolipid fractions (Lovern, 1956a and Reiser et al., 1960). The triglycerides, however, are appreciably more unsaturated in fish than in meats (Lovern,

1956b). Highly unsaturated fatty acids are quite widely distributed in small quantities as constituents of the fatty acid moiety of phospholipids (Klenk and Dreike, 1955). It is often found that the phospholipids from a particular tissue are appreciably more unsaturated than triglycerides or fats from the same source (Lea, 1957). The presence of highly unsaturated fatty acids render the lipid more susceptible to oxidation. Such oxidation is undoubtedly a major factor in deteriorative reactions leading to strongly-flavored degradative products at an early stage.

The muscle lipids of mullet begin to oxidize very rapidly after cooking, as shown by increases in TBA number and rancid odors (Zipser et al., 1962). Intensity of the reaction appears to be greater in tissues containing large quantities of lipids and heme pigments than in tissues containing less amounts. Results also show the changes in total lipid and phospholipids obtained upon cooking dark meat of mullet and storing the cooked material in the refrigerator for 5 days. A 21 percent decrease in total lipids and a 15 percent decrease in phospholipids occurred during the five days of refrigerated storage. As shown by the increase in TBA number, oxidation was very rapid during this storage period. In another similar experiment of Zipser and Watts, the decrease in total lipids was 13 percent and in phospho-

lipids 18 percent. The oxidative reaction is retarded by limited oxygen supply and by low (freezing) temperatures. Inhibition was more complete from the antioxidant mixture, sodium tripolyphosphate and sodium ascorbate, either alone or in combination with curing salts.

Chang et al. (1961) studied by means of TBA test and organoleptic evaluation the oxidation of lipids in the lean tissue of roast beef slices preserved by refrigeration, freezing or irradiation. They found that 1) oxidized products accumulate very rapidly in meat stored in the refrigerator; 2) frozen samples maintain a somewhat lower oxidation rate over long storage periods; and 3) lipid oxidation is not an important factor in irradiated beef stored at room temperature. They further observed that combinations of ascorbate and polyphosphate, used either as a dip or as a cover solution, retard lipid oxidation and greatly improve the odor of refrigerated and frozen beef. These compounds, however, do not benefit irradiated beef.

The rate of oxidation of the total milk phospholipids and of the individual phospholipid fractions was studied in model experiments with the Warburg technique by Mattsson and Swartling (1963). The results indicated that the degree of unsaturation of the phospholipid, the nature of its nitrogenous constituent and the electrolyte state of the

polar groups are of fundamental importance to the oxidation rate. Copper was found to exert a strong catalytic effect with maxima in the acid and slightly alkaline range. Hemin was an effective prooxidant at low pH and rather inactive at neutral pH. Milk phospholipids were found to oxidize most rapidly within the pH range of 2 to 4. Phosphatidylserine had the greatest oxidation rate. Phosphatidylethanolamine oxidized equally fast in the presence of copper but was very slow to oxidize without copper. Phosphatidylcholine was more resistant. Sphingomyelin did not take up any oxygen under the conditions of the model experiment.

Hornstein et al. (1961) studied the effect on flavor of lipids present in aged lean meat prior to consumption. The lipid extracted from beef and pork muscle was fractionated into triglycerides, cephalins and a mixture of lecithins and sphingomyelins. They concluded that phospholipids did not contribute to desirable meat flavor and the possibility existed that in excessively lean meat they could contribute to poor flavor.

Extraction of lipids

The primary problem is the isolation of the lipids from their biological source. There is no single standard method for lipid extraction. The method depends on the nature of

the problem and the type of biological material under study.

Many solvent systems can be employed to extract lipids. The final extract, however, should be evaporated to dryness and taken up in an appropriate solvent at the concentration of about 5 mg of lipid per ml. Phospholipids constitute about one-half to two-thirds of total lipids in most tissues of animal origin (Deuel, 1955), and can serve as a convenient indicator for approximate total lipid concentration. Folch et al. (1951, 1957) and Folch and Lees (1951) have described extraction procedures for the quantitative removal of total lipids from animal tissues. Their procedures, involving the use of chloroform-methanol as solvents, were demonstrated to remove phospholipids and proteolipids, as well as triglycerides, from fresh tissues of various animals.

Once the extract is made, it must usually be rectified to remove non-lipid material, such as amino acids, salts, urea, sugars and water-soluble phosphate esters. Various washing procedures have been used, the more common ones employing water alone and aqueous salt solutions (Folch and van Slyke, 1939; Folch et al., 1951, 1957; Bligh and Dyer, 1959; Shorland et al., 1952; Blankenhorn and Ahrens, 1955).

The lipid extracts may not necessarily require washing. It is customary in some laboratories to evaporate the first

extracts to dryness under nitrogen *in vacuo* by the use of a rotary evaporator. The resulting residue is then re-extracted with chloroform, petroleum ether, or ethyl ether. This procedure effects a partial rectification of the original extract, but some non-lipid contaminants are present. If paper chromatography is used to separate the phosphatides, such contaminants do not interfere (Marinetti *et al.*, 1957) since they remain at or near the origin on the chromatogram. The water-soluble materials may then be removed by washing the chromatogram with water, the phosphatides remaining bound to the paper. Biezenski (1962) described a simple procedure for the removal of non-lipid contaminant, from lipid extracts. The lipids are applied in a line to a silicated paper and eluted in a descending manner into a beaker with 20 percent methanol in chloroform. The impurities remain on the paper. The procedure is carried out in an anhydrous atmosphere at 30°C for 16 to 24 hours.

Degradation of the phosphatides must be minimized in order to permit the most effective use of paper and column chromatography (Folch *et al.*, 1951; Baer *et al.*, 1956; Marinetti, 1962).

Separation and identification of phosphatides

Classical methods for separating and identifying individual phospholipids (Deuel, 1957; Lovern, 1957; Wittcoff, 1951) are often laborious and seldom quantitative, and usually require large amounts of starting materials. An important advance was made in 1952 when Borgstrom reported that phospholipids could be quantitatively separated from other lipids by adsorption chromatography on a silicic acid column. In his procedure, chloroform was used to elute the non-phospholipids, and methanol to elute all phospholipids together in a single fraction. Subsequent investigators (Freeman et al., 1957; Hanahan et al., 1957; Philipps, 1958; Rhodes and Lea, 1957) have shown that further fractionation of the phospholipids is possible by elution with chloroform-methanol mixtures. In general, the silicic acid column technique has wide application. The phosphatides are separated into groups rather than into individual components. The obvious advantage of column chromatography is that it allows for the preparation of sufficient material for extensive chemical analysis.

The purification of phosphatidylethanolamine and phosphatidylserine by column chromatography has been reported by some workers (Sakagami et al., 1959; Long et al., 1960; Rouser et al., 1961). Marinetti (1962) evaluated the vari-

ous paper chromatographic methods for the identification of the phosphatides. The mobility and spot tests provide valuable information for the identification of the chromatographic components. These paper chromatographic methods become extremely informative when coupled with column chromatography and other techniques available for identification of organic compounds e.g., infrared analsis, gas liquid chromatography, nuclear magnetic resonance, mass spectrometry, spectrophotometry, and chemical analysis.

Microgram quantities of tissue phospholipids are commonly determined by phosphorous analysis (Chen et al., 1956; Harris and Popat, 1954; Bartlett, 1959). The various procedures involve the digestion of organic material and the colorimetric determination of inorganic phosphate. An average molecular weight (MW) of 750 for the phospholipids is assumed for converting the inorganic phosphate to phospholipids.

Infrared spectrophotometry has been introduced to analyze the intact phospholipid molecule rather than its hydrolysis products. In 1957, Schwarz et al. used a combination of infrared and chemical analyses to study numerous small fractions obtained from chromatography of brain lipids and rabbit-skin phospholipids. Nelson and Freeman

(1959) described a simplified method, using infrared spectrophotometry, to determine cephalins, lecithins, and sphingomyelins in relatively small samples of human serum.

Smith and Freeman (1959) claimed that a combination of chromatography and infrared spectrophotometry is useful for studying milk phospholipids. Chromatographic fractionations can be followed and the principal components readily determined from the spectra. Accuracy is influenced by variations in the extinction coefficients of various lecithins, etc., and by the composition of the fractions examined, but is probably equal to that attained by microchemical analysis (Nelson and Freeman, 1959; Schwarz et al., 1957). Only comparatively small samples are necessary for infrared spectrophotometry and they may be recovered if desired. Isolation and quantitative analysis of constituents occurring in minor amounts, such as lysophospholipids, inositol-containing phospholipids and plasmalogens, would require both a more elaborate fractionation procedure and highly purified reference compounds.

MATERIALS AND METHODS

Preparation of turkey tissues

Fresh tissue samples of white and red muscle, liver and heart were obtained from nine 1-year-old Keithley white turkey hens. In the second group, twelve 16-week-old toms were used in obtaining the various tissues plus the gizzard. These birds were divided into three groups of four each. Each group was then stored for 0, 60 or 180 days at -25°C. Duplicate moisture determinations were done on each tissue sample along with the total lipid and phospholipid analyses.

Extraction of lipids from tissues

The extraction procedure was essentially the same as that of Folch et al. (1957). Twenty grams of ground tissue were blended for 2 minutes with cold chloroform-methanol mixture (2:1 v/v) in proportion of 10 ml of solvent mixture per gram of tissue (except 20 ml of solvent per gram of liver tissue) in a Waring blender. The slurry was immediately filtered and the clear filtrate mixed with 0.2 of its volume of 0.03 M magnesium chloride in a separatory flask. The mixture was allowed to stand overnight at -5 to -7°C. Upon standing, a biphasic system was obtained, with essentially all of the tissue lipids in the lower phase and

non-lipid materials in the upper phase. The lower phase was drained out and accurately measured. An aliquot was taken and evaporated in an oven at 50°C to get the weight of the total lipid.

Purification of the lipids

Various lipids have been separated and purified by adsorption on and displacement elution from a silicic acid column. Application of the silicic acid column to separate complex lipid mixtures has been made by a number of workers and the techniques used in this study are modifications of the methods of Borgstrom (1952), Fillerup and Mead (1953), Hanahan (1957) and others.

Phospholipid content was determined by three different methods: gravimetric analysis, analysis of the phosphorous content in the various fractions isolated, and from standard curves established from IR spectrum (Kuchmack and Dugan, 1963). The contents of phosphatidylethanolamine, phosphatidylserine, and lecithins as determined by these three different methods are generally in good agreement.

In general, it is unnecessary to carry out all the above analyses. A combination of paper chromatography of the intact phosphatide with any one of the above techniques can identify the major phosphatides of a given tissue. It is understood that this approach may not be unequivocal

and that it will identify only the type of phosphatide, such as lecithin, phosphatidylethanolamine, etc. It will not reveal the number of molecular species present in the sample. This can only be approximated by a careful and detailed analysis of the fatty acids, aldehydes and others present.

Silicic acid column A column (12 x 180 mm) constructed for this work was fitted with a 100 ml reservoir on the top. A nitrogen supply was connected by rubber tubing to the column through a ground glass ball and socket joint on top of the reservoir. A glass wool plug was placed at the bottom to support the silicic acid. Five grams of silicic acid (Mallinckrodt AR 100-mesh) heated overnight at 120°C were slurried with chloroform and poured onto the column. Air bubbles were removed by stirring the mixture with a long glass rod. The silicic acid was allowed to settle and the chloroform to drain under slight nitrogen pressure. The column, after washing with 30 ml of chloroform was ready to use. Approximately 30 to 40 mg of lipid sample in chloroform were carefully layered on the top surface of the column and again a slight nitrogen pressure was applied. The sample was allowed to drain nearly to the surface of the silicic acid. Additional small quantities of solvent were

added slowly until the sample was completely transferred to the column.

Separation of non-phosphorous lipids from phospholipids

Anhydrous chloroform was used to wash the column to which the lipid sample was added. This procedure displaced all of the neutral lipids and free fatty acids while the phospholipids remained adsorbed on the silicic acid. The neutral lipids and fatty acid fractions were dried in the oven at 50 to 60°C and weighed.

Separation of phospholipids The phospholipids were eluted from the columns with the following successions of solvents: 1) 80 ml chloroform-methanol (4:1 v/v); 2) 100 ml chloroform-methanol (1:1 v/v); and 3) 150 ml methanol.

Large samples of each fraction were separated and collected in a round bottom flask. The volume was concentrated on a rotary evaporator for use in paper chromatography and IR reading. In a duplicate run on the column, the small samples were separated and collected in test tubes with Packard Automatic fraction collector (Model 230). Approximately 3-ml samples were collected in 20 x 150 mm tubes. Each sample was analyzed for phosphorous by the method of Chen et al. (1956), and for the number of phospholipid components by chromatography on silicic acid-impregnated paper (Marinetti et al., 1957 and Marinetti, 1962).

A follow-up run was done using more combinations of solvents to find out if the minor components present in each fraction above would be clearly separated from the major ones. The solvents used were: 1) 50 ml chloroform-methanol (19:1 v/v); 2) 50 ml chloroform-methanol (9:1 v/v); 3) 80 ml chloroform-methanol (4:1 v/v); 4) 80 ml chloroform-methanol (3:2 v/v); 5) 80 ml chloroform-methanol (1:1 v/v); 6) 50 ml chloroform-methanol (1:4 v/v); and 7) 50 ml methanol.

Phosphorous determinations

Phosphorous determination was carried out by pipetting an aliquot of the total lipid extract which contained about 5 to 125 ug P or 100 ug to 2.5 mg phospholipid into a 20 x 150 mm Pyrex test tube. The solvent was removed by evaporation at 60 to 70°C in an oven, and 1 ml of the acid mixture (70 ml perchloric acid, 20 ml nitric acid and 10 ml of sulfuric acid) was added. A micro-Kjeldahl apparatus was employed for digestion (Zipser et al., 1962). The samples then were digested for 30 minutes to 1 hour to get complete combustion and clear solution. After cooling the tubes, 0.5 ml of 8 percent sodium hydroxide was added around the top inner surface in order to rinse the walls. The samples were made to volume with water (10 to 25 ml) to give 1 to 3 ug of P per ml. Two ml of the aqueous dilutions were

placed in a tube and 2 ml of the freshly prepared color reagent were added and mixed. The color reagent was prepared by mixing 2 parts of distilled water, 1 part of 10 percent ascorbic acid, 1 part of 6N sulfuric acid and 1 part of 2.5 percent ammonium molybdate (Chen et al., 1956). The samples or fractions and a standard (0 to 2.5 ug P/tube in duplicate) were heated in a water bath at 38°C for 1½ hours to develop the color. The samples and standard were read at 820 mu on the Beckman DU spectrophotometer.

Qualitative tests on paper chromatograms

The paper chromatography of the various fractions, as well as the intact total lipid samples, was done on silicic acid-impregnated paper (Marinetti et al., 1957 and Marinetti, 1962).

Silicic acid-impregnated paper Sodium silicate was prepared according to the method of Marinetti (1962) by first dissolving 147.5 g sodium hydroxide in 250 ml of water. This solution was added, with stirring, to a suspension of silicic acid (Mallinckrodt analytical reagent, 100-mesh) in water (155 g in 400 ml) which contained 0.85 g soluble starch. Starch was added to the sodium silicate to eliminate some of the powdery nature of the impregnated paper (Marinetti, 1962). The sodium silicate was cooled and diluted to 850 ml for use.

Impregnation of filter paper Whatman No. 1 filter paper was cut into 14 x 23 cm sheets. Larger sheets were used initially but proved to be very difficult to handle when wet. The sheets were immersed in the sodium silicate solution for 5 minutes, hung vertically to drain for 5 minutes (Hack, 1961). A dozen sheets were prepared at one time. Contact between the newly-dipped paper and the papers in the acid bath had to be avoided until the silicate on the paper had a chance to react with the hydrochloric acid (10 to 15 seconds); the paper was immersed in the bath with the other papers. Silicic acid-impregnated papers were washed, after decanting the hydrochloric acid, for 30 minutes in running tap water and in distilled water for 30 minutes. The flow of water was kept to a minimum to prevent leaching of silicic acid from the paper. The papers were hung to dry and then pressed between glass plates overnight to remove wrinkles (Marinetti, 1962). The finished papers were stored in a plastic bag to avoid absorption of vapors.

Paper chromatography of phospholipids In applying lipids to the paper, a pencil line was drawn 2 cm from the bottom of the paper to locate the origin. The side used depended on whether the solvent was to travel parallel to the 14 or 23 cm edge of the paper. Phosphatides were applied

in volumes of about 10 to 30 ul at 2 to 3 cm intervals along the pencil line. The concentration of phosphatide determined the quantity added since over-loading caused very poor separation and resolution.

Development of chromatograms The papers to which samples had been added were rolled into cylinders and the ends held together with cotton thread. Care was taken to prevent the vertical ends of the paper from touching each other. The paper cylinder was developed in a 15 x 24 cm Mason jar by ascending chromatography using the diisobutylketone-acetic acid-water (40:20:3 v/v) solvent of Marinetti and Stotz (1956).

Rhodamine 6-G test for all phosphatides Staining with Rhodamine 6-G is a general test for all phospholipids and permits their separation into two groups: 1) the acidic phosphatides such as inositol phosphatides, serine phosphatides and phosphatidic acids are colored lilac or blue (Rouser et al., 1956); and 2) the neutral phosphatides, sphingomyelins, lecithins, and ethanolamine phosphatides are stained yellow or orange.

The paper chromatograms were dried in a hood at room temperature for one hour and then immersed for 2 to 3 minutes in Rhodamine 6-G solution (12 mg per liter) 4 to 5 minutes (Marinetti et al., 1957). The excess dye was rinsed

off with distilled water and wet chromatograms were viewed under ultra-violet light (366 mu).

Ninhydrin test for amino-phosphatides The dry chromatograms were dipped once into a 0.25 percent ninhydrin solution in acetone-lutidine, 9:1 (v/v). Lutidine (or collidine) must be used or the color reaction is very weak. After the papers were dried at room temperature, the amino lipids appeared as purple spots on a white background. The test is quite specific for phosphatides having a free amino group.

Qualitative test by infrared analysis of phosphatides

Samples of phosphatides eluted from the silicic acid column were evaporated and redissolved in chloroform. Infrared measurements were made with a Perkin Elmer, Model 21, infrared spectrophotometer.

Oxygen uptake measurements using the Warburg apparatus

The oxidation of total lipid and of the various phospholipid fractions was determined by measuring the oxygen uptake in a GME-Lardy Warburg apparatus, Model WB-4.

The Warburg apparatus has been widely used in the field of biological chemistry to study autoxidation reactions of various types, including a few studies on milk phospholipids, (Mattsson and Swartling, 1963) and fatty

oils (Johnston and Frey, 1941).

The equipment permits exact control of experimental conditions, which must be rigidly reproduced if good precision is obtained.

The Warburg equipment and technique are described by Dixon (1934) and Umbreit et al. (1957). The apparatus used had two types of reaction flasks: one was conical with a center well and one sac provided with a ground joint venting plug, capacity of 15 ml; and the other one was conical with center well and two sacs, one with a venting plug, the other with ground glass stopper, capacity of 16 ml.

Preliminary work indicated that with 0.3 to 0.5 mg of phosphorous (approximately 10 to 20 mg of sample), a test period of 54 hours was necessary to show a "plateau effect" with fraction II (lecithin). Although this time was not sufficient for other fractions to plateau, the same test period was used. The test was initiated by introducing the dissolved sample into the vessel. Following removal of the solvent with nitrogen, the flask was then attached to the manometer (or the flask was fitted tightly with a cork and held at -30°C if the measurement could not be made at once).

The flask-manometer assembly was then flushed with nitrogen at a pressure of 15 to 20 cm of Brodie solution for 10 minutes. This was immediately followed by a stream

of oxygen at a pressure of 10 to 15 cm of Brodie solution for 5 minutes and then equilibrated for 10 minutes before sealing the system to begin pressure measurements. The manometers were set at 15 cm during equilibration. Pressure readings were taken at 30-minute intervals during the first 3 to 4 hours to estimate the induction period and at various intervals thereafter. In essentially all cases, the customary definition of the induction period is the time required for the absorption rate to become rapidly accelerated.

The experiments were carried out at 40 $\pm$ 0.02°C in an atmosphere of air and with an oscillation rate of 80 per minute, at an amplitude at 5 cm.

The apparatus used in this study included ten manometers and flasks, two sets being used as thermomanometers. Thus a maximum of four samples in duplicate were measured at one time.

The vessels were cleaned of the oxidized lipids by rinsing with chloroform. They were then immersed in hot soapy water for at least 3 hours, followed by successive washings of distilled water, cleaning solution, distilled water and methanol, and then dried at 100°C.

Package treatment of turkeys Measurements of oxygen uptake by total lipid and phospholipid fractions were done on samples from packaged and unpackaged whole turkeys.

Four Keithley white male turkeys were used in this study. Two were vacuum-packaged with Cryovac bags and the other two were stored without a package. They were frozen at -30°C for 48 hours, then transferred to a -16°C freezer where they were held for 17 days. Samples of pectoralis major muscle were removed from each side of the breast on which total lipid extraction was done.

Cooked and uncooked turkey Oxygen uptake was measured on total lipid, phospholipid and non-phospholipid fractions of cooked and uncooked samples. Two Keithley white turkey males 10 months old, and weighing 25 to 28 pounds eviscerated, were used in this experiment. Fresh samples of pectoralis major muscle were removed from one side of the breast by making a slit in the skin. The skin was then drawn over the opening and stitched back in place before the turkey was wrapped in aluminum foil and roasted at 200°C to an internal temperature of 80°C.

After the turkey had been cooked, a sample of pectoralis major was taken from the other side of the breast. A total lipid extract was prepared from both cooked and uncooked samples from the same turkey.

RESULTS AND DISCUSSION

Total lipid and phospholipid

A preliminary investigation of the average lipid and phospholipid contents of the various tissues from nine one-year-old turkey hens are presented in Table 1. In white meat (breast), the phospholipid in percent of the total lipid is less than in red meat (leg). This was also observed between white and red chicken meat from the data of Kaucher et al. (1943). It is interesting to note, as pointed out by Bloor (1943), that higher phospholipid content is associated with muscles of greater activity. He found that heart muscle, in general, is most active and contains the most phospholipid on the average. He also found that thigh muscle of the domestic rabbit contained only 1.7 percent phospholipid as contrasted with 3.75 percent in the muscle of the wild rabbit. This may explain why leg muscle of turkey or chicken has higher phospholipid compared to the breast muscle. In Tables 2 and 3 are presented the percent total lipid and phospholipid from tissues of different species reported by previous workers.

Table 1. Average total lipid and phospholipid in turkey tissues

Turkey	Phospholipid % of tissue		Total lipid % of tissue		Phospholipid % of total lipid
1 yr. old hen (9)	Wet	Dry	Wet	Dry	
White meat	1.11	4.11	3.31	11.94	33.53
Red meat	1.27	4.93	3.20	12.28	39.69
Liver	4.54	16.70	5.64	18.80	80.50
Heart	2.40	10.41	3.89	16.89	61.70

Table 2. Average total lipid and phospholipid in various fresh tissues

Species	Phospholipid	Total lipid	P.L.% of T.L.	References
Pig fetus	0.59			Gortner, 1945
Fish				
Cod	0.35-0.85			
Haddock	0.17-0.48			
Salmon	0.85			Lovern, 1956a
Sardine	0.35-0.43			
Livers of these	0.16-2.00			
Pig heart	0.75	2.83		Marinetti et al. 1957
Ox heart	1.30			Gray and Macfarlane, 1958
Rabbit				
Skeletal muscle	0.51	2.26	23	Gray and Macfarlane, 1961

Table 2 (Continued)

Species	Phospho-lipid	Total lipid	P.L.% of T.L.	References
Pigeon				
Breast muscle	2.22	5.16	43	
Heart muscle	2.55	3.86	66	
Trout				
Muscle 1	0.63	5.31	12	
Muscle 2	0.70	3.72	19	
Pig				
Spleen	1.24	2.45	50	
Lung	1.59	2.00	79	
Kidney	2.34	2.90	81	
Beef (Stored 10 days)	1.00	4.57	21.88	Hörrstein et al. 1961
Pork	0.75	6.61	11.35	
Mullet				
White meat		1.74		Zipser et al. 1962
Red meat		6.65		
Raw (0 days)	0.93	8.22	11.31	
Cooked and stored:				
0-days	0.97	7.64	12.70	
3-days	0.83	6.85	12.12	
5-days	0.79	6.52	12.11	
Rat liver	1.75-2.48			Harris and Gambal, 1963
Hog meat	0.50			Kuchmack and Dugan, 1963
Mouse		4.25-5.81		Camejo et al. 1964
Rat		3.68-4.69		
Rabbit		2.75-3.31		
Guinea pig		2.63-3.25		
Pigeon breast		5.17	28	Davenport, 1964
Ox longissimus dorsi		1.60	24	

Table 3. Average total lipid and phospholipid in dry muscle or tissues[a]

Muscle	Total phospholipid (%)	Total lipid (%)
Veal	5.04	11.84
Lamb	4.74	11.19
Salmon	4.39	18.30
Chicken (Red)	4.36	12.67
Chicken (White)	2.72	6.95
Codfish	4.28	9.47
Shrimp	3.89	8.12
Pork	3.06	22.40
Beef	3.08	14.21
Liver	16.22	22.99
Heart	9.83	16.45

[a]From data of Kaucher et al., 1943.

Interestingly, muscles which differ widely in physiology may be similar in composition. Bloor (1943) reported that gizzard and stomach muscles have an average phospholipid content and fatty acid content of 2.6 and 1.9 percent, respectively, based on dry weight. Moreover, he noted that the iodine number of the phospholipid fatty acids

showed no constant difference in the much-used or little-used muscles.

The function of the phospholipids as discussed by Sinclair (1934) are: 1) as possible oxygen-transporting substances due to the ability of their double bonded carbons to take up and probably to transfer oxygen; 2) as intermediaries in the metabolism of the fats, which Sinclair regards as certain in the early stages of fat absorption and transport, but doubtful in the later stages of combustion in the tissues. However, more recently accepted views are that phospholipids act: 1) as structural elements in tissues; 2) in the transport of fat into cells owing to the solvent action of the phospholipid on fat; and 3) in mitochondrial electron transfer and in oxidative phosphorylation (Green and Fleischer, 1963).

The foregoing statements point out possible functions of phospholipids and suggest that high phospholipid content would be useful to highly active muscle. If phospholipid transports oxygen, the "active" muscle would have two or three times the oxygen-carrying substance as the "inactive" muscle. As Whipple (1926) has shown, it has also 2 or 3 times as much muscle hemoglobin, which is also an oxygen carrier. In active muscle, the presence of increased phospholipid intermediates, or of substances useful in fat transport, would be expected.

In general, redness and lipid content of muscle are associated. Muscles with the highest lipid content, such as heart, jaw, and neck are invariably deep red. The pectoral muscles of the pigeon are deep red, those of the chicken are white, and the phospholipid content of the pigeon pectoral muscles is 3 times that of the hen. On the other hand, the lipid content of the gizzard muscle of birds and of the stomach muscles of the rabbit is the same, although the gizzard muscle is dark red and the stomach muscle is pinkish or white. The thigh muscle of beef is red in color, while its lipid content was among the lowest found (Bloor, 1927). Later, Bloor and Snider (1934) reported a low correlation between redness of muscle and its phospholipid and cholesterol contents.

Table 1 also shows that organ tissues have higher phospholipid content than muscle tissues. The average value for liver phospholipid is 16.70 percent of the dry tissue, compared to the heart, 10.41 percent. Kaucher *et al.* (1943) reported average values of 16.22 percent and 9.83 percent in dry liver and heart tissues, respectively. Organ tissues such as liver, heart, kidney and lung contain relatively large amounts of non-fat lipids. These tissues, like brain, contain more phospholipid than all other essential lipid combined. Bloor *et al.* (1930) pointed out that tissues highest in essential lipids are those possessing

the greatest extent and variety of physiological functions. For example, the physiological function of liver is very diversified as compared to muscle in which essential lipid is much lower.

In the next group of turkeys studied (Table 4), the values were taken from four, sixteen-week-old toms stored 0-days, another group stored 60-days and the last group stored 180 days at -25°C. Although the one-year-old hens had higher total lipid contents in white and red muscles, their percent phospholipid in total lipid was lower than that of the younger toms. This difference is largely due to the fat or glyceride content. Kaucher et al. (1943) reported that young tissue has more essential lipid and less fat than older tissue.

The phospholipid of dry tissue showed a decreasing trend with storage. The differences were statistically significant ($P \geq .01$). Analyses of variance for phospholipid and total lipid are presented in the Appendix. Similarly, the decrease of phospholipid as a percent of total lipid with storage was significant ($P \geq .05$). However, the total lipid of dry tissue did not show a significant decrease with storage. These results suggest that there are some apparent changes in phospholipid composition during storage. Zipser et al. (1962) reported a 21 percent decrease in total lipids and a 15 percent decrease in phospholipids in the dark meat of mullet

Table 4. Average total lipid and phospholipid in turkey tissues stored for various periods at -25°C

Turkey	Phospholipid % of tissue		Total lipid % of tissue		Phospholipid % of total lipid
16 wk. old tom (4) (Stored 0 days)	Wet	Dry	Wet	Dry	
White meat	.58	2.21	1.00	3.81	59.50
Red meat	1.02	4.38	1.60	6.88	64.45
Liver	5.21	18.85	6.02	21.79	85.50
Heart	2.70	12.57	2.93	13.64	90.50
Gizzard	.84	3.91	1.35	6.22	63.00
16 wk. old tom (4) (Stored 60 days)					
White meat	.48	1.85	1.07	4.15	46.95
Red meat	.94	4.02	1.74	7.41	54.38
Liver	4.43	15.31	5.06	17.61	87.50
Heart	2.06	9.34	2.99	13.51	69.25
Gizzard	.79	3.76	1.50	7.04	52.00
16 wk. old tom (4) (Stored 180 days)					
White meat	.53	1.95	1.32	4.85	41.00
Red meat	.87	3.39	1.79	7.04	42.94
Liver	3.03	10.66	4.70	16.59	64.25
Heart	1.84	8.26	2.56	11.46	71.88
Gizzard	.86	3.87	1.45	6.53	59.50

after five days of refrigerated storage. As shown by the increase in TBA number, oxidation was very rapid during this storage period. In another experiment of Zipser et al. (1962), the decrease in total lipids was 13 percent and in phospholipids 18 percent. Oxidative reactions were retarded by limited oxygen supply and by low (freezing) temperatures.

The values obtained in Tables 1 and 4 are in good agreement with the values reported by previous workers from tissues of different species (Tables 2 and 3).

Quantitative column chromatography

Although paper chromatography is admirably suited for work on a microscale, it does not permit easy isolation of sufficient material for a more complete analysis. Hence, in order to further the investigation on the turkey tissue phosphatides, column chromatography on silicic acid was employed. The phosphatides were fractionated by solvents containing increasing amounts of methanol in chloroform.

Removal of non-phosphorous-containing lipid All of the neutral fats: mono-, di- and tri-glycerides, free fatty acids and cholesterol esters were apparently eluted in the initial chloroform fraction, which also contained most of the pigments, but essentially no phosphorous. In Table 5, the average values of non-phosphorous lipid, in mg per g

Table 5. Distribution of non-phosphorous lipids and individual phospholipids[a] in turkey tissues

	Breast	Leg	Liver	Heart	Gizzard
Non-phosphorus lipids (mg/g of wet tissue)	4.13	6.96	11.51	11.37	6.79
% of total lipid	41.69	52.11	25.92	38.15	44.38
Total phospholipid (mg of P/g of wet tissue)	0.24	0.34	1.15	0.85	0.40
Cephalin	30.41[b] (34.54)[c]	32.78 (36.47)	34.56 (28.45)	42.81 (30.38)	32.73 (46.53)
Lecithin	59.47 (51.64)	54.70 (51.18)	49.27 (57.57)	38.55 (52.58)	49.51 (42.20)
Sphingomyelin	1.98 (14.31)	2.50 (14.77)	3.51 (13.98)	6.43 (17.03)	6.89 (11.27)
% recovery	104.09	106.41	96.22	97.22	99.38

[a]Based on phosphorous analysis, and expressed as a percentage of total phosphorous in the sample.

[b]7-solvent system.

[c]3-solvent system.

of wet tissue, and its percent in total lipid were taken from a separate group of turkey males stored at -25°C for about 150 days. The data also show that total lipids of organ tissues, particularly in the liver or heart, have less neutral lipids or non-phosphorous lipids compared to the muscle tissues of breast, leg or gizzard. The presence of more pigments in leg muscle may be a possible explanation for the higher non-phosphorous lipids in leg rather than in breast muscle.

Kuchmack and Dugan (1963) stated that there was a wide variation in the content of non-phospholipids in meat. Removal of depot fat when taking a muscle sample from the ham or loin was rather simple, but on the other hand, because of fine overlapping of fat and muscle tissue on the rib or belly, they found that separation of fat was more difficult, and variation in neutral fat content was expected.

Separation and identification of individual phospholipids Lipid samples from different turkey tissues were examined by column chromatography, paper chromatography and infrared analysis. Column chromatography consistently gave four major peaks of phosphorous concentrations, as shown in Figs. 1 through 5 for the various tissues. Paper chromatography likewise gave 4 major spots (2 to 5) as shown in Fig. 6.

Fraction I The material from fraction I was eluted with 4:1 chloroform-methanol (v/v). It consistently

Fig. 1. Chromatography of turkey breast phospholipids on silicic acid column. Major fractions: A, cephalin; B, phosphatidyserine; C, lecithin; and D, sphingomyelin

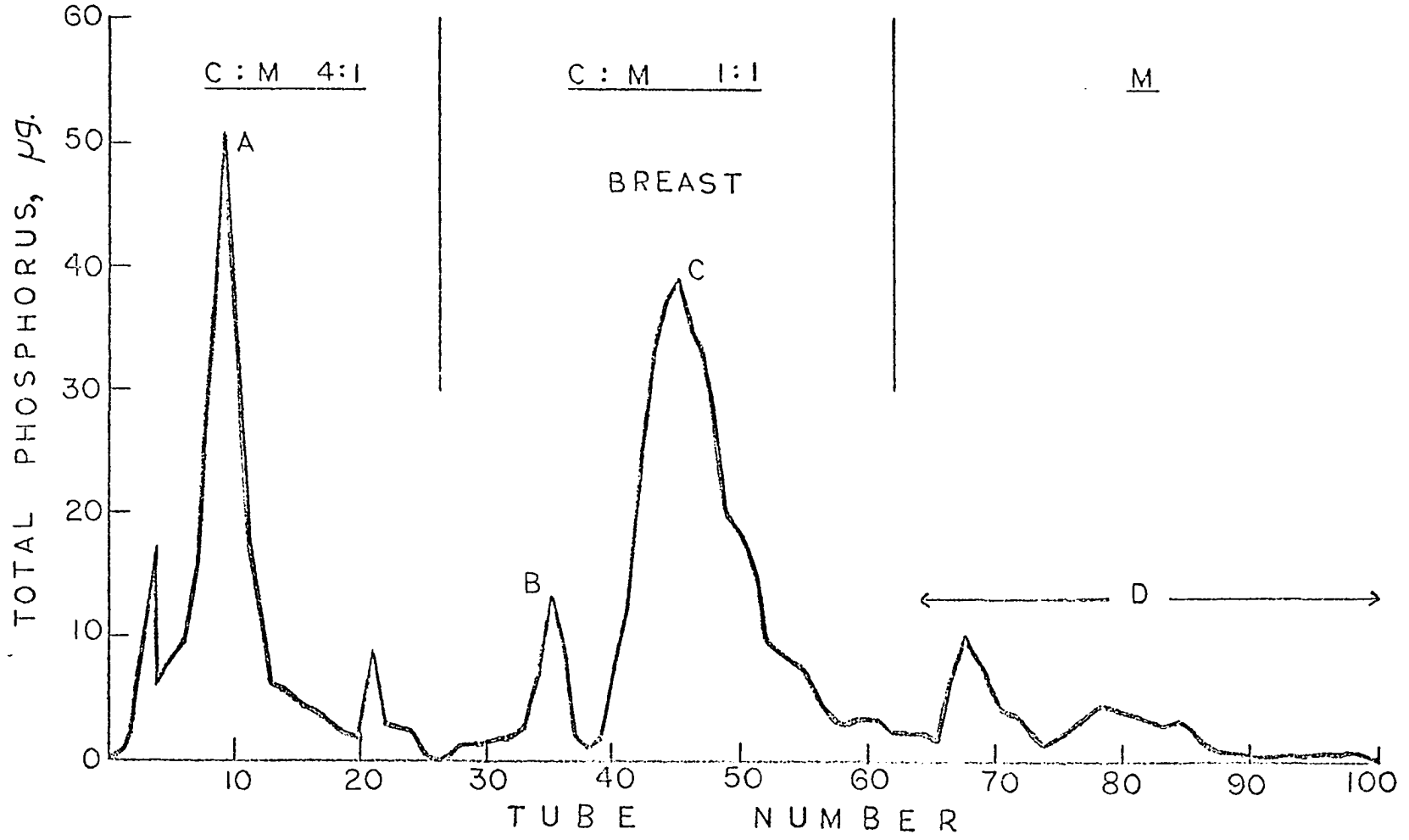

C : M 4:1
C : M 1:1
M
BREAST
A
B
C
D
TOTAL PHOSPHORUS, µg.
TUBE NUMBER
0
10
20
30
40
50
60
70
80
90
100

Fig. 2. Chromatography of turkey leg phospholipids on silicic acid column. Major fractions: A, cephalin; B, phosphatidyserine; C, lecithin; and D, sphingomyelin

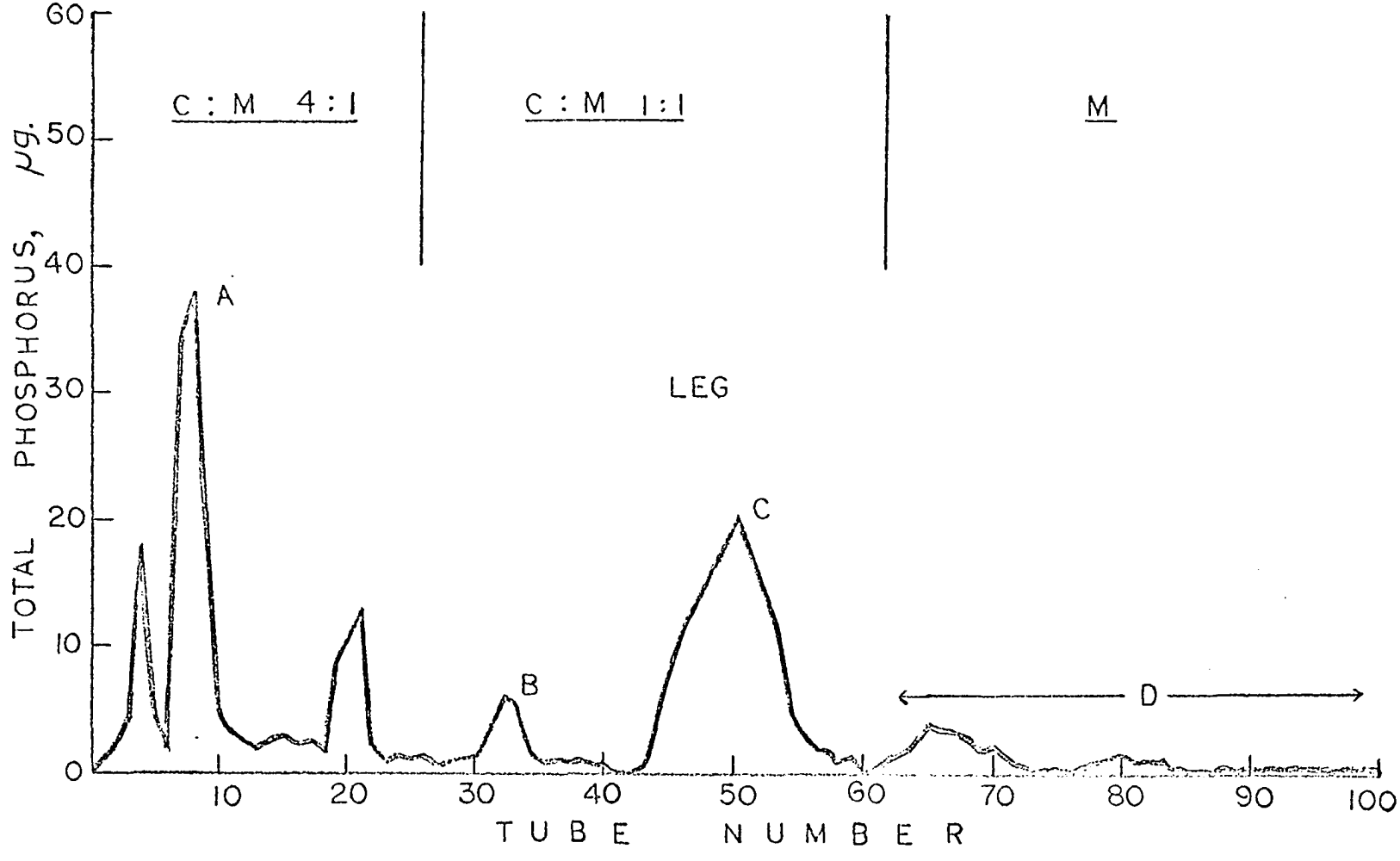

C : M 4 : 1
C : M 1 : 1
M
LEG
A
B
C
D
TOTAL PHOSPHORUS, µg.
0
10
20
30
40
50
60
10
20
30
40
50
60
70
80
90
100
TUBE NUMBER

Fig. 3. Chromatography of turkey liver phospholipids on silicic acid column. Major fractions: A, cephalin; B, phosphatidylserine; C, lecithin; and D, sphingomyelin

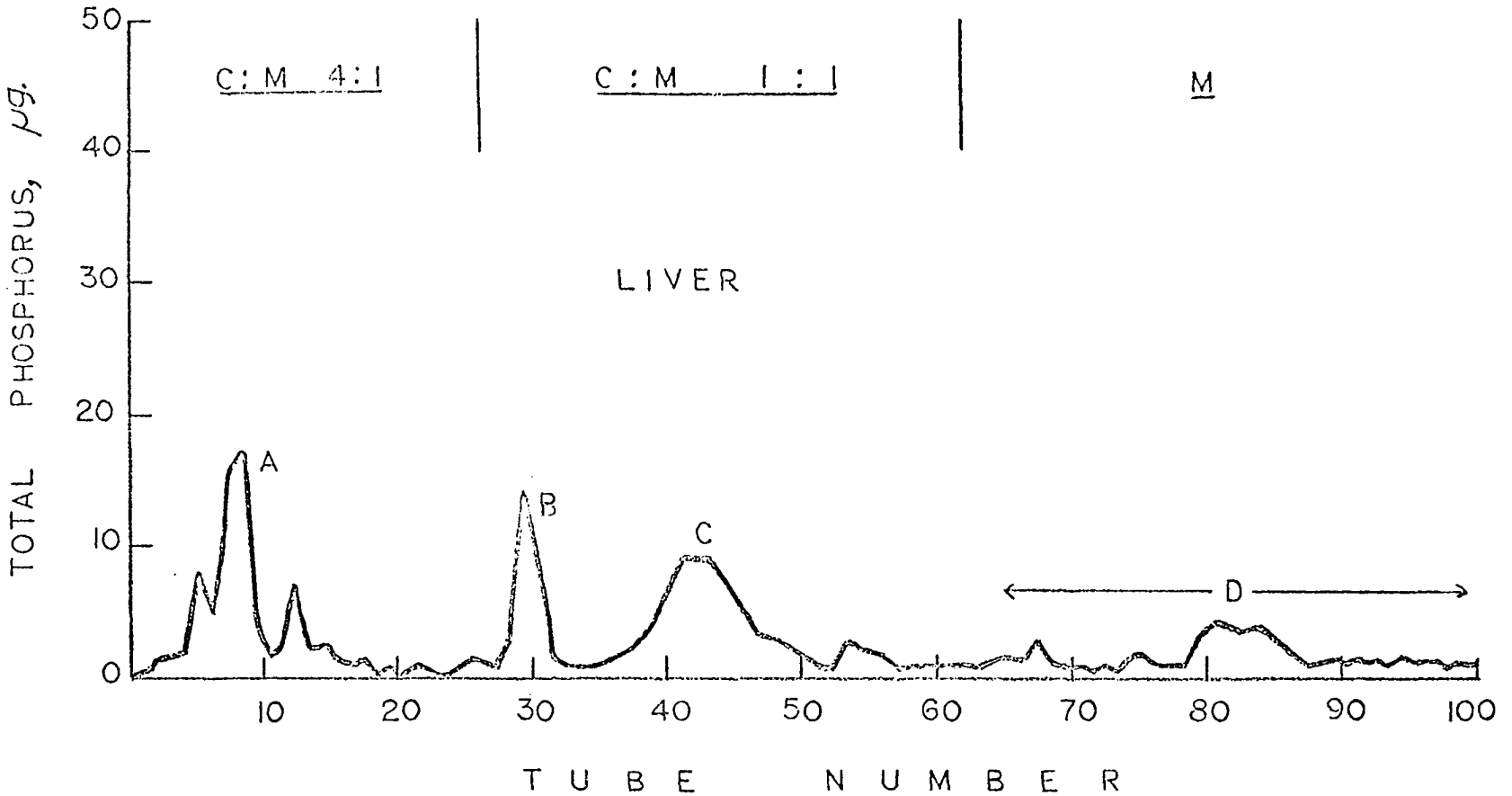

C : M 4 : 1
C : M 1 : 1
M
LIVER
TOTAL PHOSPHORUS, µg.
50
40
30
20
10
0
A
B
C
D
10
20
30
40
50
60
70
80
90
100
T U B E N U M B E R

Fig. 4. Chromatography of turkey heart phospholipids on silicic acid column. Major fractions: A, cephalin; B, phosphatidylserine; C, lecithin; and D, sphingomyelin

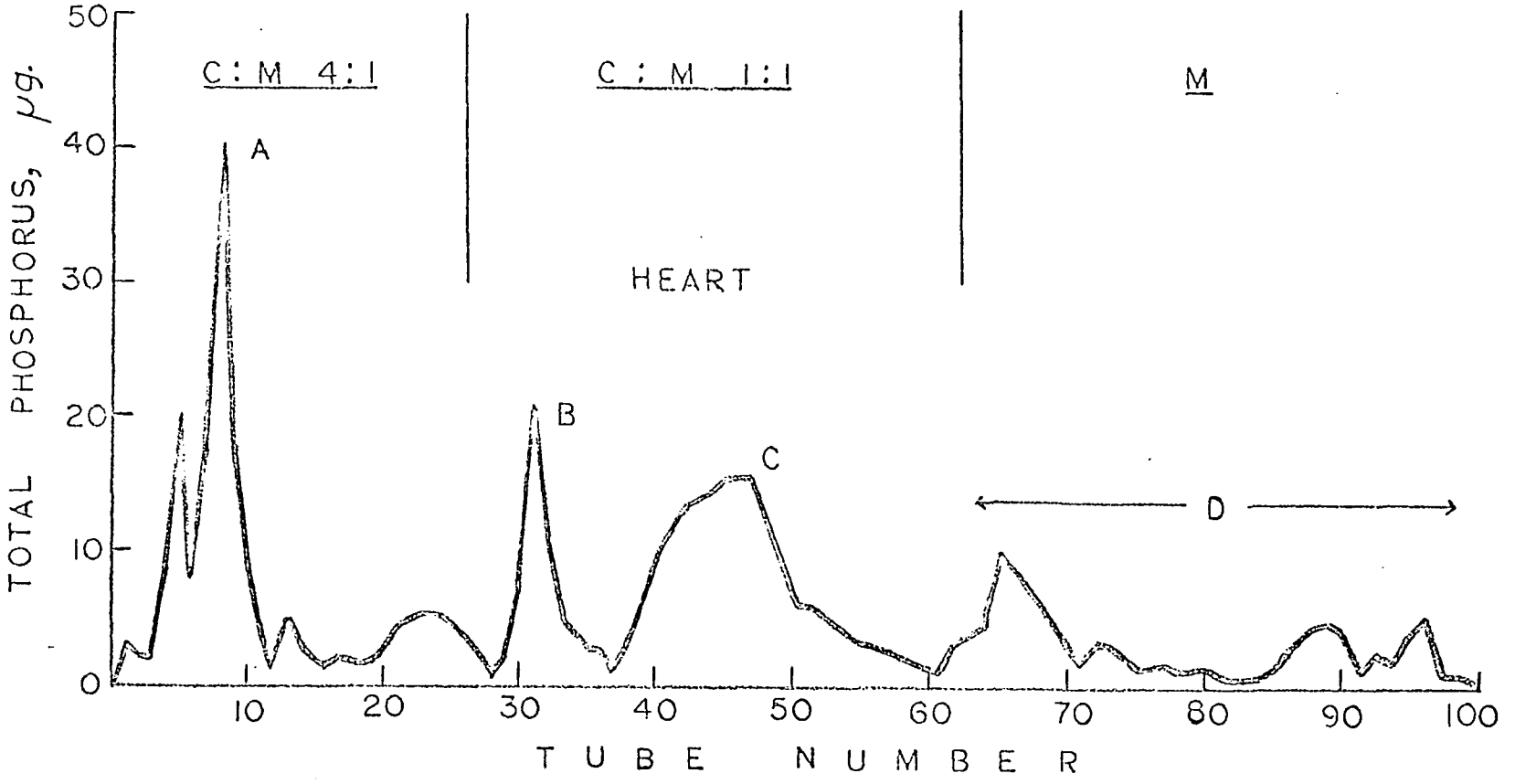

TOTAL PHOSPHORUS, µg.
50
40
30
20
10
0
C : M 4:1
C : M 1:1
M
HEART
A
B
C
D
10
20
30
40
50
60
70
80
90
100
TUBE NUMBER

Fig. 5. Chromatography of turkey gizzard on silicic acid column. Major fractions: A, cephalin; B, phosphatidylserine; C, lecithin; and D, sphingomyelin

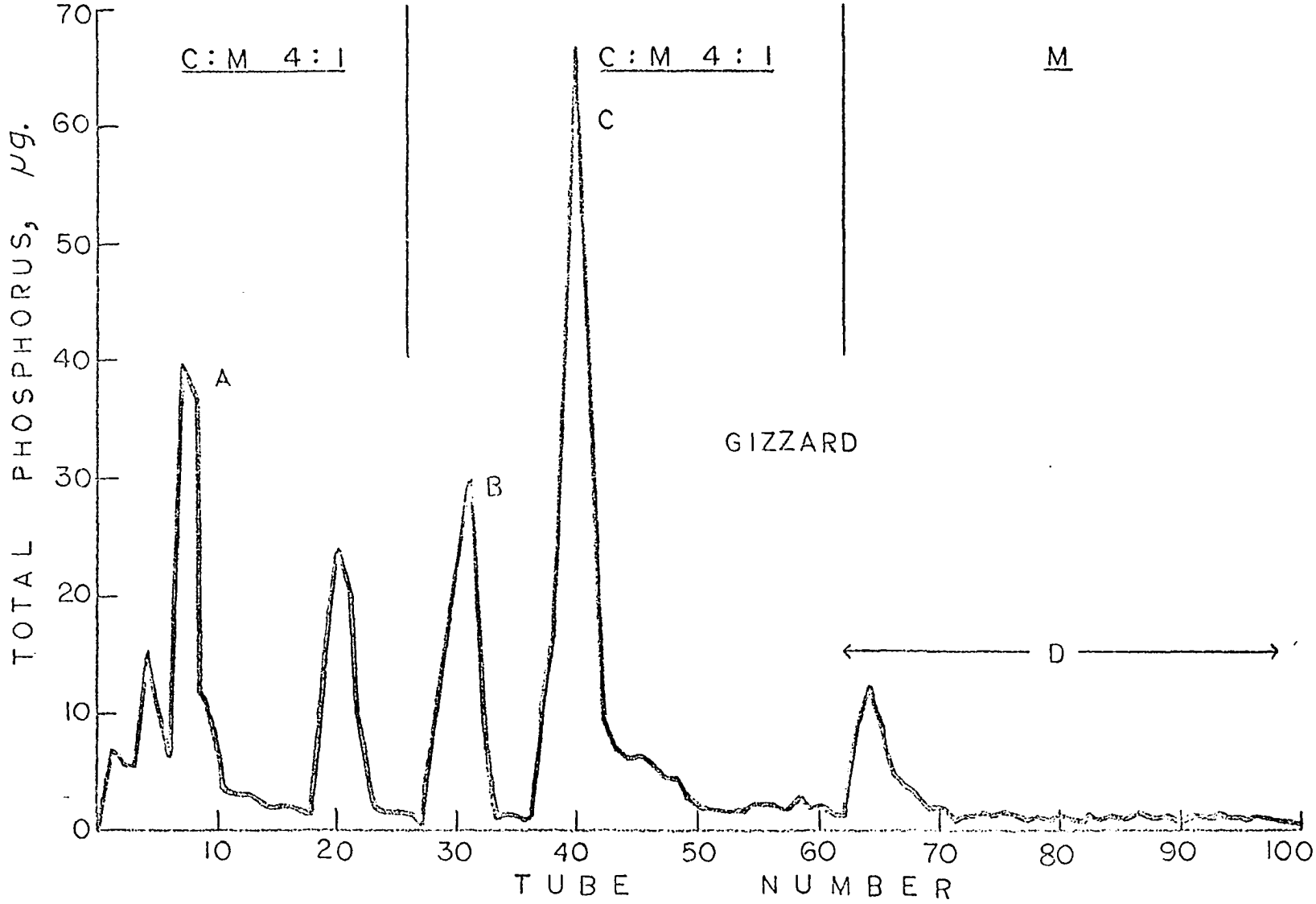
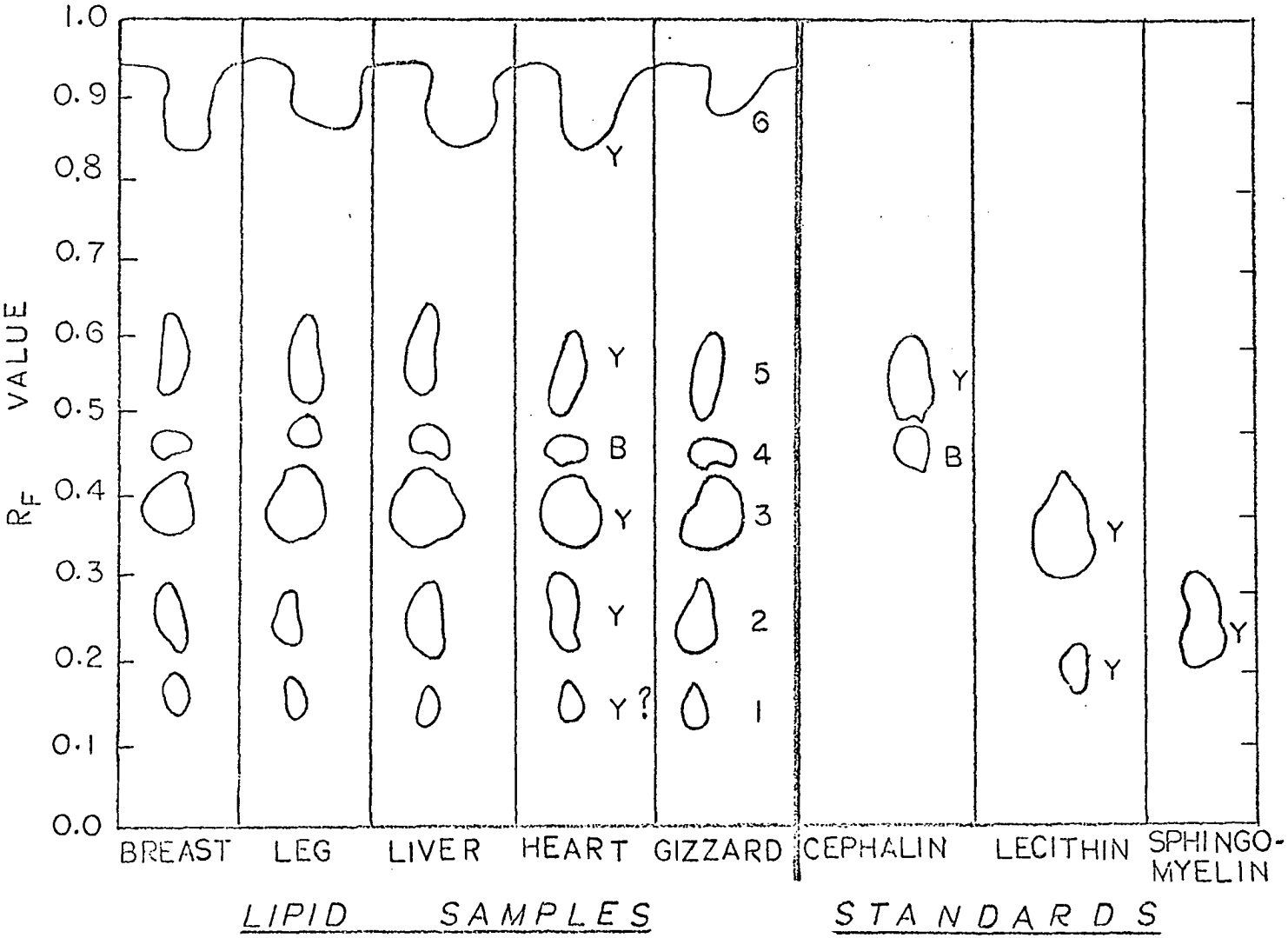

C : M 4 : 1
C : M 4 : 1
M
A
B
C
D
GIZZARD
TOTAL PHOSPHORUS, µg.
TUBE NUMBER
0
10
20
30
40
50
60
70
80
90
100

Fig. 6. Paper chromatogram of phospholipid standards, and of turkey tissue phospholipids. Each spot was identified as: 1) possibly lysolecithin; 2) sphingomyelin; 3) lecithin; 4) phosphatidylserine; and 5) cephalin. Abbreviations: Y, yellow; B, blue


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
R_F VALUE
Y
6
Y
5
B
4
Y
3
Y
2
Y?
1
Y
B
Y
Y
Y
BREAST
LEG
LIVER
HEART
GIZZARD
CEPHALIN
LECITHIN
SPHINGO-MYELIN
LIPID SAMPLES
STANDARDS

showed one major peak (A) and two other minor ones eluted before and after the main peak. Paper chromatography of a concentrate of fraction I produced one main ninhydrin-reacting spot (Fig. 6) and a second very weak spot (often times not visible). The major spot (5) turned yellow to Rhodamine 6-G stain method and the second spot (4) turned very faint blue to Rhodamine 6-G stain. Their average Rf values, 0.52 and 0.45 respectively, correspond to the Rf values of cephalin standards (see also Table 6).

The infrared spectra of fraction I (Fig. 7) indicated that it was mainly a cephalin fraction. Notably, there is an absence of the band at 10.3 u which is exhibited by both lecithin and sphigomyelin. Phosphatidylethanolamine and phosphatidylserine are believed to exhibit similar or identical infrared spectra (Smith and Lowry, 1962; and Smith and Freeman, 1959).

Fraction II The fraction II material, which was eluted with 1:1 chloroform-methanol (v/v) consisted of two peaks, B and C. The minor peak consistently came off ahead of peak C (Fig. 1). On paper (Fig. 6), a very faint spot (4) moved ahead of the main spot (3). It showed a very weak ninhydrin spot with an average mobility of 0.45. The main spot turned yellow to Rhodamine 6-G had an Rf value of 0.39 which correspond to 0.40, Rf value of the lecithin standard.

Table 6. Characteristics of phosphatide components of turkey tissues as compared to the standards

Spot No.	Rf value	Rhodamine 6-G stain	Ninhydrin stain	Probable identity of component
1	.17	Yellow (W)[a]	-	Unidentified
2	.30	Yellow (M)	-	Sphingomyelin
3	.39	Yellow (S)	-	Lecithin
4	.45	Blue (W)	W	Phosphatidylserine
5	.52	Yellow (S)	S	Phosphatidylethanolamine
6		Yellow (S)	-	Neutral lipids
Stds:				
Cephalin	.45 (.45)[b]	Blue (W)	W	Phosphatidylserine
	.52 (.50)	Yellow (S)	S	Phosphatidylethanolamine
Lecithin	.20 (.18)	Yellow (W)	-	Lysolecithin
	.40 (.37)	Yellow (S)	-	Lecithin
Sphingomyelin	.26 (.31)	Yellow (S)	-	Sphingomyelin

[a]Abbreviation: W, weak; M, moderate; S, strong; -, negative.

[b]Rf values given here are approximate values as obtained by Marinetti, 1962.

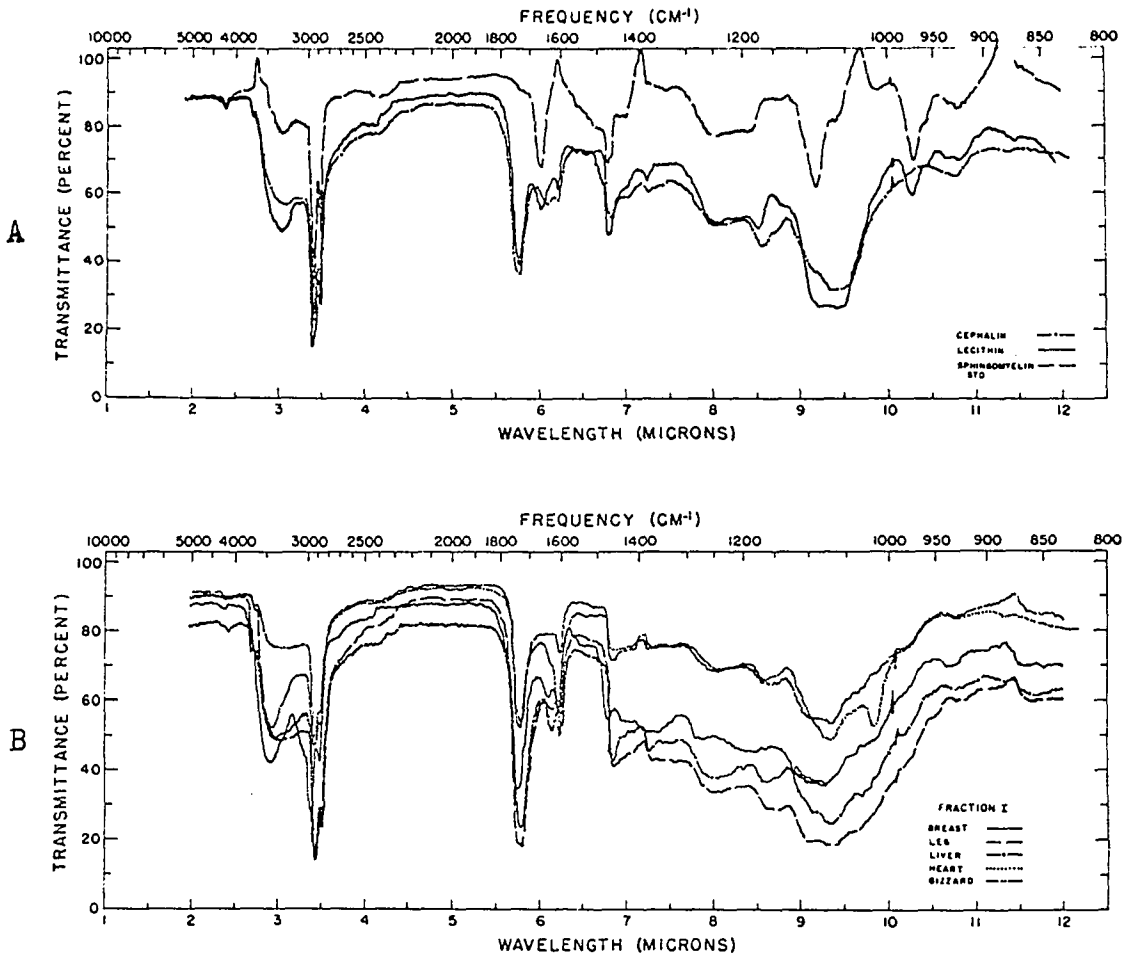


Fig. 7. Infrared spectra of (A) PL standards, and (B) fraction I (cephalin) from various turkey tissues

When this whole fraction was analyzed for its infrared spectra, it showed a strong band at 10.3 u (Fig. 8). This fraction, therefore, appeared to be mainly lecithin (peak C), and followed the minor peak (B), phosphatidylserine portion and/or phosphatidyl inositol.

The phosphatidyl inositol fraction isolated from the silicic acid column was eluted largely with chloroform-methanol (3:2 v/v) as expected from the work of Hanahan et al. (1957), but some was found in the chloroform-methanol (4:1 v/v) eluate as well. Work by those researchers indicated that phosphatidyl inositol from liver has the same column chromatographic characteristics as phosphatidyl inositol from soybean, wheat germ and brain. All of the preparations were similar according to thin-layer chromatography, paper chromatography, and by infrared spectrophotometric examination, although the preparation from the liver showed two spots on paper and thin-layer chromatography.

Fraction III Fraction III material was eluted with methanol (Fig. 1) and produced one main peak (D). Comparison with a sphingomyelin standard on paper chromatography (Fig. 6) showed it to have similar mobility (spot 2) and staining reaction with Rhodamine 6-G stain method. The main component of this fraction, therefore, appeared to be sphingomyelin. The unidentified spot (1) which trailed the sphingomyelin must be lysolecithin, as evidence by the Rf

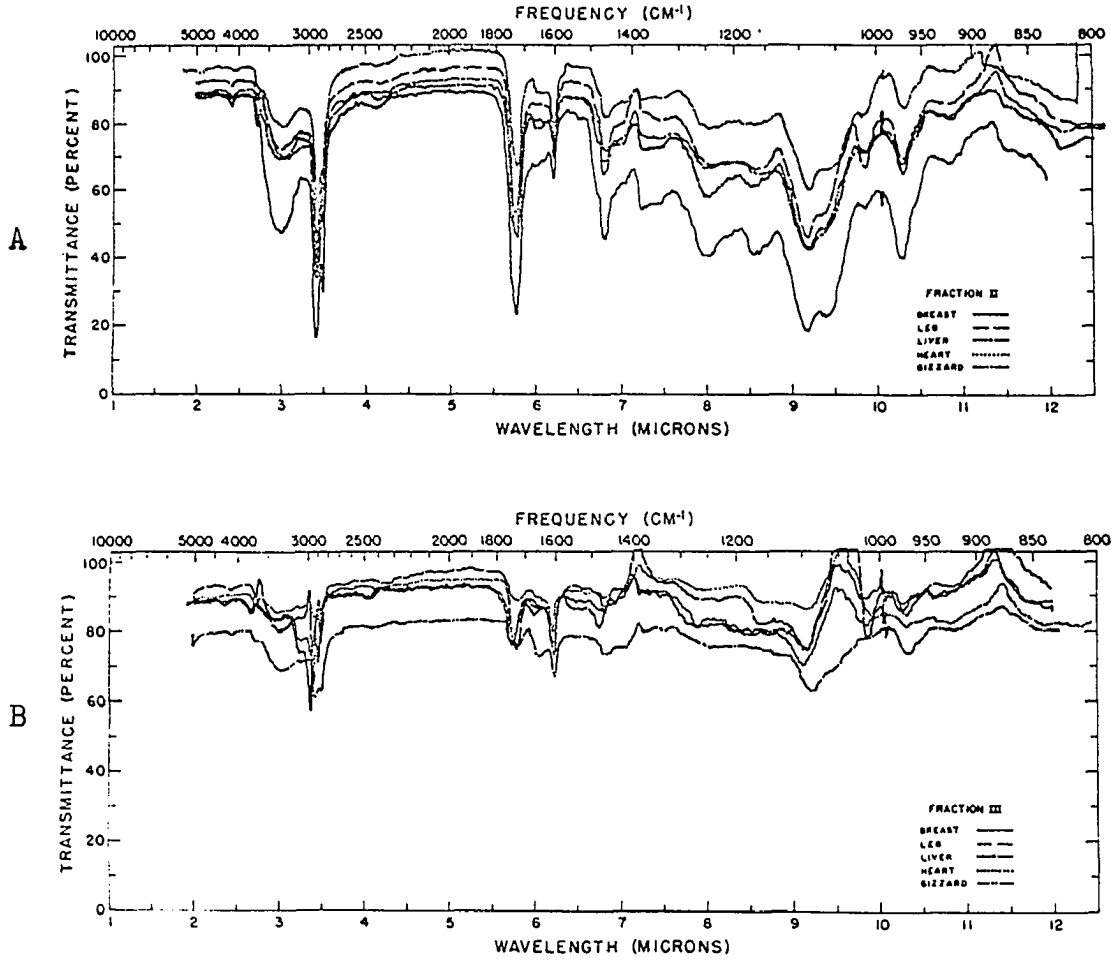


Fig. 8. Infrared spectra of (A) fraction II (lecithin), and (B) fraction III (sphingomyelin) from various turkey tissues

value (Marinetti, 1962).

The infrared analysis of the fraction showed a strong band at 6.1 to 6.3 u (Schwarz et al., 1957 and Smith and Freeman, 1959), which is comparable to that of sphingomyelin. This spectrum actually represents sphingomyelin with some admixture of lecithins and lysolecithin.

As can be seen, some of the fractions are heterogenous in composition. Indeed, the column chromatography concentrated some components and thereby made possible their detection. Figs. 9 through 13 show a general elution curve of the phosphatides from turkey tissues using a 7-solvent system. Table 5 (see page 38) shows the approximate distribution of the three major phospholipids in turkey tissue based on the two sets of solvent combinations. The amount of the individual major phospholipid varies with the solvent system used. For example, the percent cephalin in the 7-solvent system was calculated mainly from peak A which was eluted with 4:1 methanol-chloroform, eliminating the minor components which came off ahead with smaller increments of methanol. The minor components were included in the total amount of cephalin with the 3-solvent system.

The elimination of these minor constituents which came off with 5 and 10 percent methanol reduced the total phospholipids (based on total phosphorous) to less than 100 percent compared with those obtained in the 3-solvent system.

Fig. 9. Chromatography of turkey breast phospholipids on silicic acid column. Major fractions: A, cephalin; B, phosphatidylserine; C, lecithin; and D, sphingomyelin

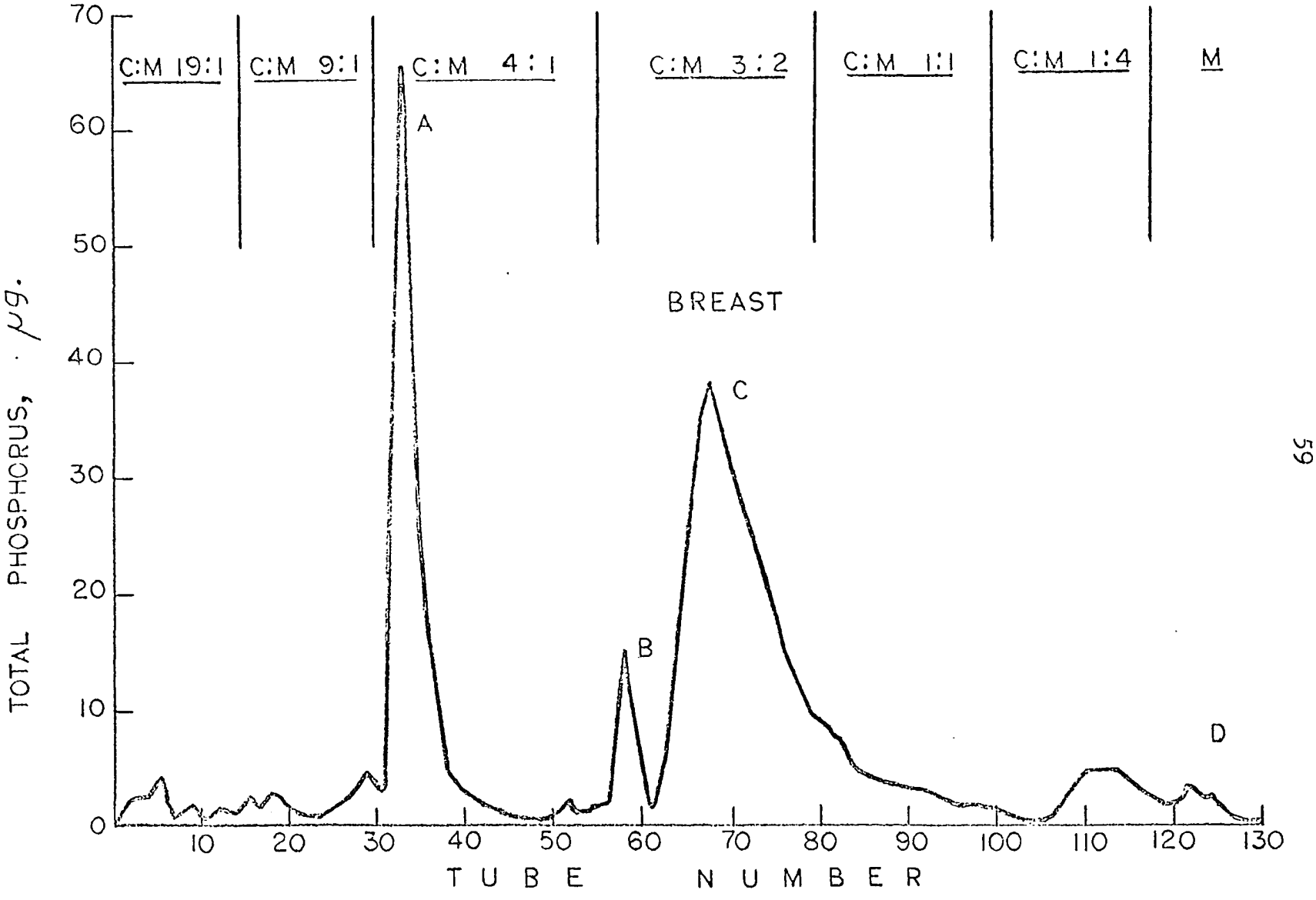

C:M 19:1
C:M 9:1
C:M 4:1
C:M 3:2
C:M 1:1
C:M 1:4
M
A
B
C
D
BREAST
TOTAL PHOSPHORUS, µg.
0
10
20
30
40
50
60
70
80
90
100
110
120
130
TUBE NUMBER

Fig. 10. Chromatography of turkey leg phospholipids on silicic acid column. Major fractions: A, cephalin; B, phosphatidylserine; C, lecithin; and D, sphingomyelin

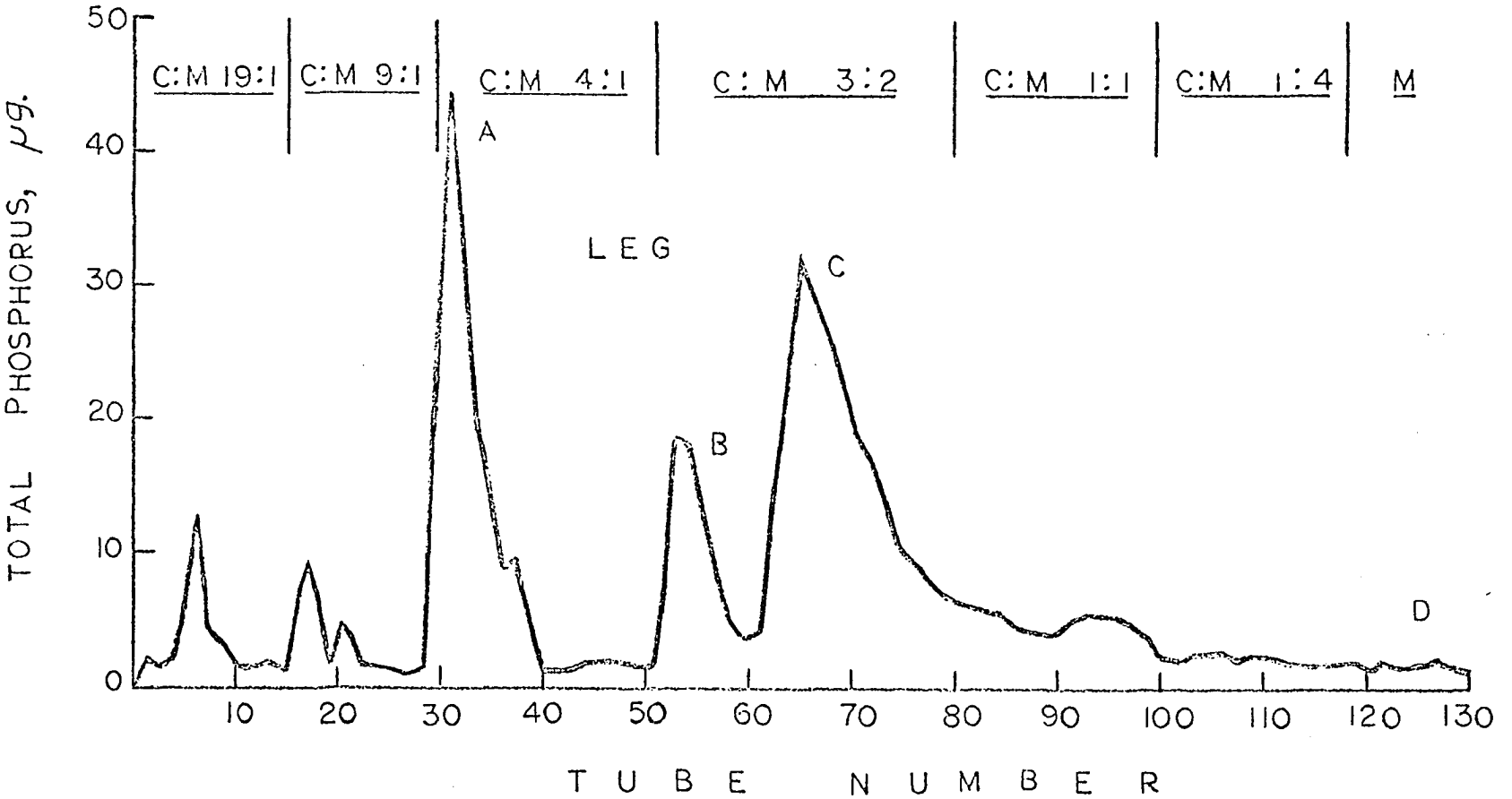

C:M 19:1
C:M 9:1
C:M 4:1
C:M 3:2
C:M 1:1
C:M 1:4
M
A
B
C
D
LEG
TOTAL PHOSPHORUS, µg.
0
10
20
30
40
50
10
20
30
40
50
60
70
80
90
100
110
120
130
TUBE NUMBER

Fig. 11. Chromatography of turkey liver phospholipids on silicic acid column. Major fractions: A, cephalin; B, phosphatidylserine; C, lecithin; and D, sphingomyelin

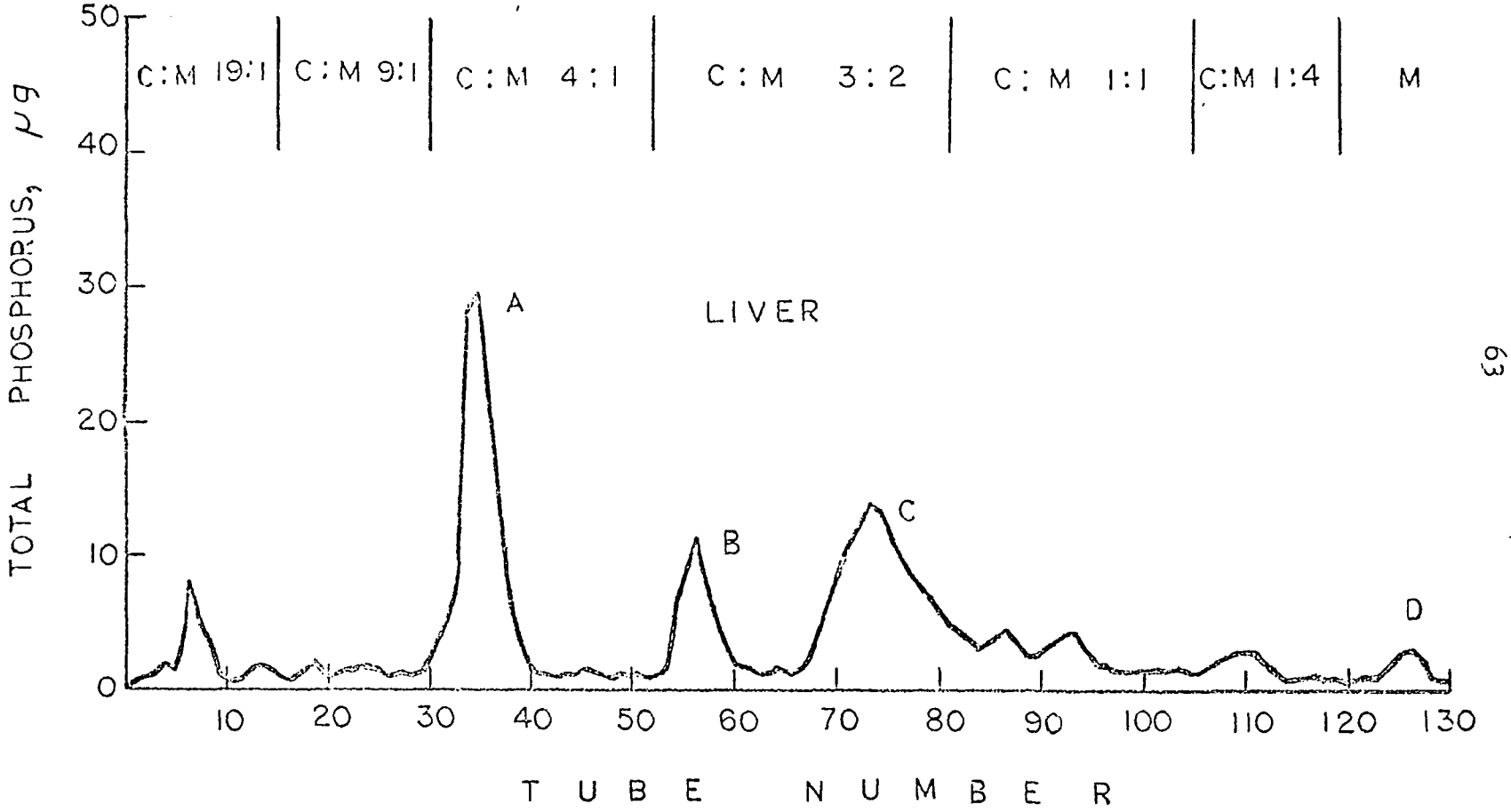

C:M 19:1
C:M 9:1
C:M 4:1
C:M 3:2
C:M 1:1
C:M 1:4
M
TOTAL PHOSPHORUS, μg
50
40
30
20
10
0
A
B
C
D
LIVER
10
20
30
40
50
60
70
80
90
100
110
120
130
TUBE NUMBER

Fig. 12. Chromatography of turkey heart phospholipids on silicic acid column. Major fractions: A, cephalin; B, phosphatidylserine; C, lecithin; and D, sphingomyelin

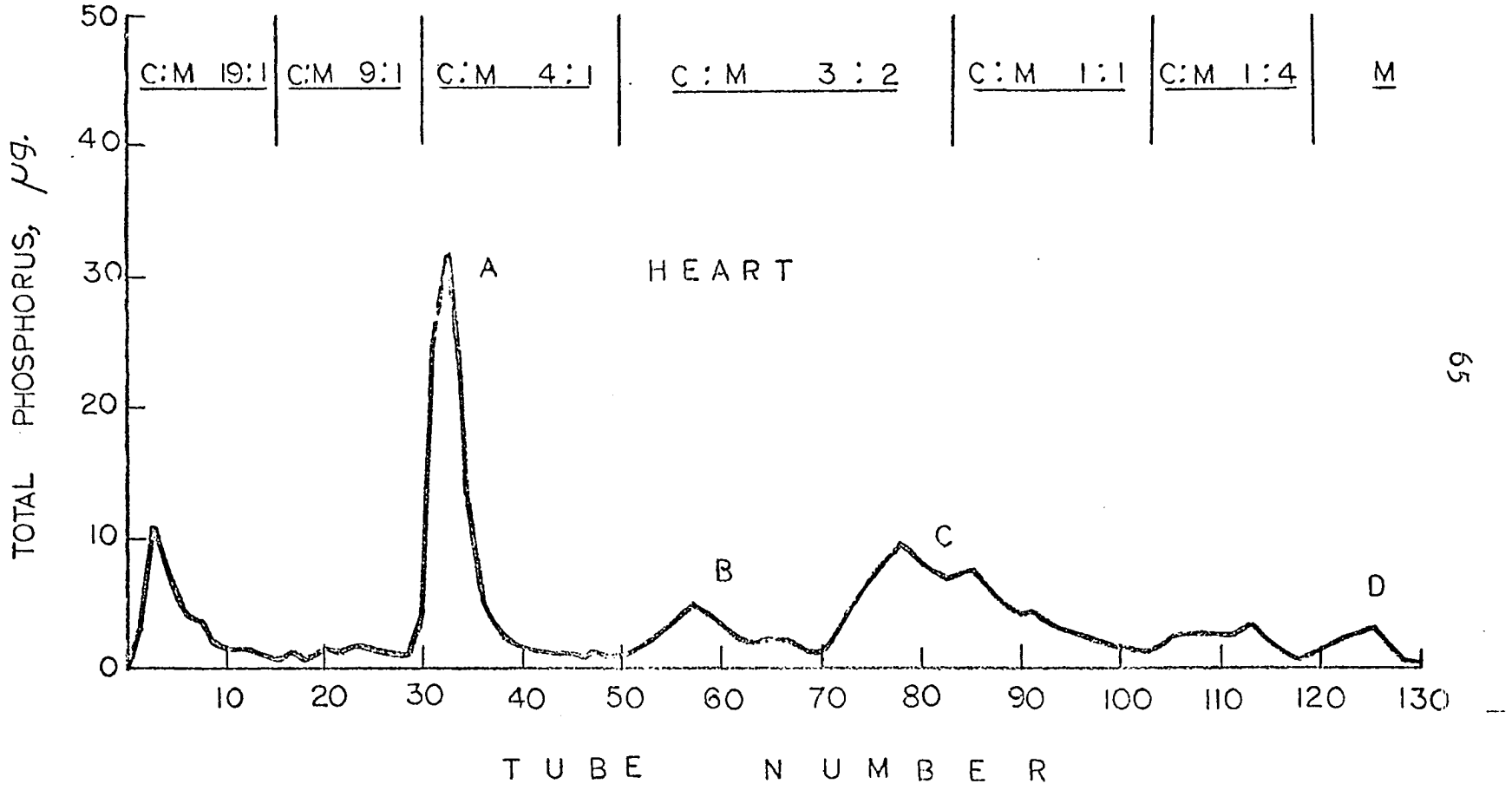
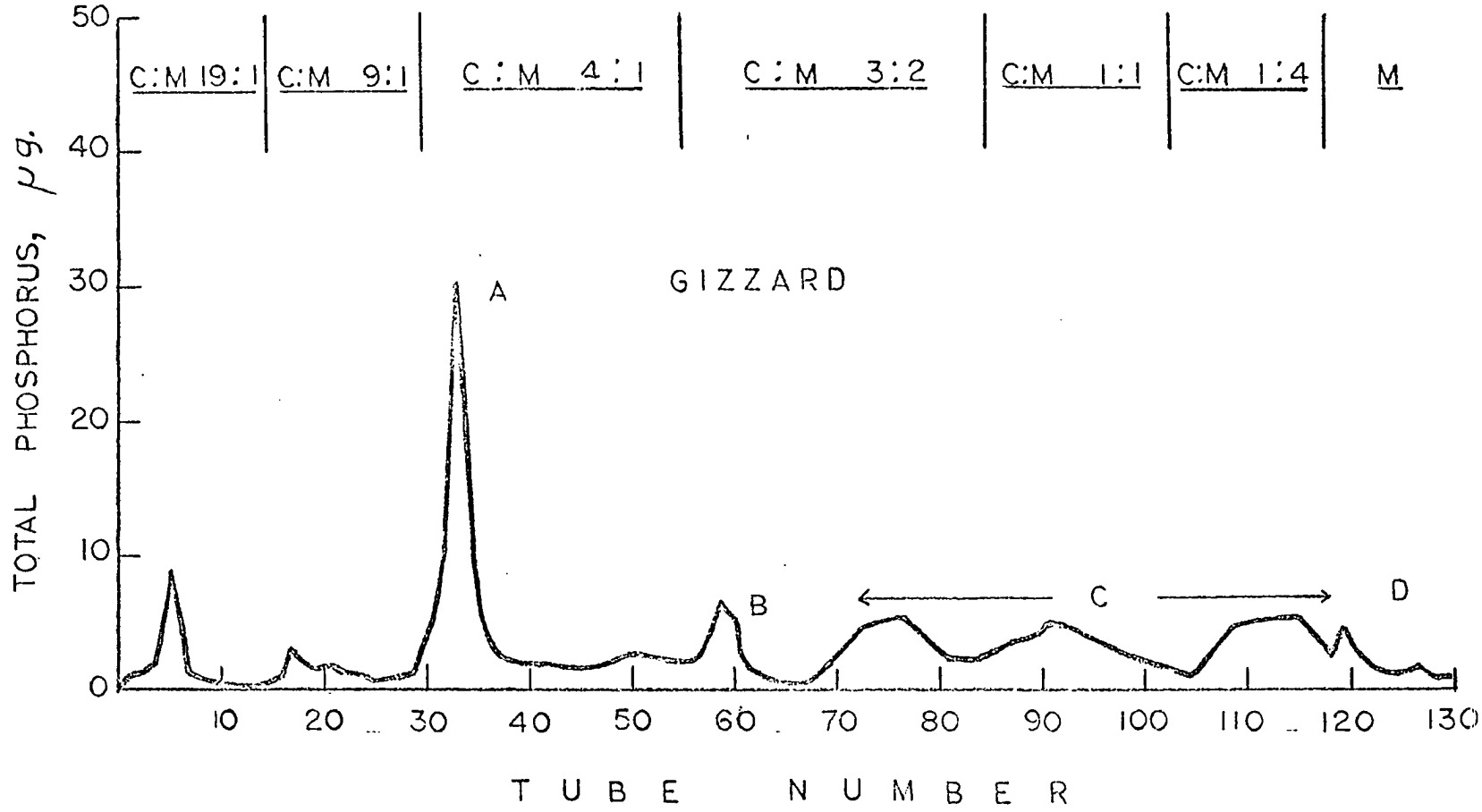

C:M 19:1
C:M 9:1
C:M 4:1
C:M 3:2
C:M 1:1
C:M 1:4
M
HEART
A
B
C
D
TOTAL PHOSPHORUS, µg.
0
10
20
30
40
50
10
20
30
40
50
60
70
80
90
100
110
120
130
TUBE NUMBER

Fig. 13. Chromatography of turkey gizzard phospholipids on silicic acid column. Major fractions: A, cephalin; B, phosphatidylserine; C, lecithin; and D, sphingomyelin


C:M 19:1
C:M 9:1
C : M 4 : 1
C : M 3:2
C:M 1:1
C:M 1:4
M
GIZZARD
A
B
C
D
TOTAL PHOSPHORUS, µg.
0
10
20
30
40
50
10
20
30
40
50
60
70
80
90
100
110
120
130
TUBE NUMBER

Note that peak B (Fig. 9) never resolved from lecithin (peak C) in the 7-solvent system. From the Rf measurements this was believed to be a portion of phosphatidylserine of the cephalin fraction, and/or phosphatidyl inositol. From the weak positive reaction to ninhydrin, this might also be either a lysocephalin or a different kind of amino-containing phosphatide. Since no further attempt was made to study this minor constituent, it was considered as a minor component of the lecithin fraction.

On the other hand, peak D which was believed to be mainly spingomyelin with traces of lecithin and lysolecithin has been resolved from the lecithin peak (C), hence the amount has decreased as shown in Table 5.

It is interesting to note that the organ tissues, liver and heart, had higher lecithin values and lower cephalin values in contrast to muscle tissue of breast, leg and gizzard in the 3-solvent system (Table 5). The opposite was found with the 7-solvent system.

No values have been reported in the literature on phospholipids of turkey tissues. However, results from this study are comparable, in general, with the values reported by several workers on tissue of different species, as shown in Table 7.

The present findings are to be compared with the results obtained by others using silicic acid column chromatography.

Table 7. Components of phospholipids as percent of total phospholipids of various fresh tissues

Species	Cephalin[a]	Lecithin	Sphingomyelin	References
Fish				
Cod	12	77	11	Lovern, 1956a
Salmon	42	58	nil	
Pig heart	33.51	55.8	10.1	Marinetti et al., 1957
Ox heart	43	42	5	Gray and Macfarlane, 1958
Sheep				
Skeletal muscle	25.10	42.20	6.4	Dawson, 1960
Liver	31.10	55.20	4.2	
Heart muscle	29.30	38.90	6.6	
Rabbit				
Skeletal muscle	24	65	-	Gray and Macfarlane, 1961
Pigeon				
Breast muscle	46	49	3	
Heart muscle	29	49	5	

[a]The term cephalin includes phosphatidylethanolamine, phosphatidylserine, and the corresponding acetal phosphatides.

Table 7 (Continued)

Species	Cephalin[a]	Lecithin	Sphingomyelin	References
Trout				
Muscle	25	66	2	
Pig				
Spleen	43	35	19	
Lung	31	51	12	
Kidney	41	39	14	
Beef Pork	40-45	40-45	10-15	Hornstein et al., 1961
Ox heart	29.90	41.70	-	Dawson et al.,
Ox liver	17.20	55.70	5.8	1962
Rat liver	37.8	48	9.0	Webster and Thompson, 1962
Hog muscle				
Belly	37.5	58.6	3.9	Kuchmack and
Ham	42.0	54.7	3.3	Dugan, 1963
Loin	38.0	60.8	1.2	
Rib	30.9	63.0	6.1	

Hanahan et al. (1957) fractionated beef liver lipids on silicic acid columns and recognized the presence of phosphatidylethanolamine, phosphatidylserine, phosphoinositide, lecithin, and sphingomyelin in column eluates. Neither paper nor thin-layer chromatography was used. Since these investigators washed the lipid with acetone, some differences in composition from the crude chloroform-methanol extracts examined in this laboratory can be expected.

Getz et al. (1961) studied the lipid composition of rat liver by silicic acid chromatography in much the same manner as Hanahan et al. (1957).

Nelson (1962) studied the lipids of mouse liver by silicic acid chromatography and examined column fractions by thin-layer chromatography. Aside from neutral lipids, he noted the presence of phosphatidylethanolamine and serine, monophosphoinositide, lecithin, lyso-lecithin and sphingomyelin.

These previous investigators did not encounter the very large number of lipid components observed in the study of Rouser et al. (1963). Usually investigators observed 5 to 7 components and occasionally noted the possible presence of cardiolipin. The studies of Rouser et al. (1963) disclosed some 30 or more polar lipids in beef liver when all definitely different spots on thin layer chromatograms, prepared from diethylaminoethyl (DEAE) column fractions, were counted.

Some of the lipids appear to be new classes since they were eluted from the DEAE columns with solvents that differentiate them from known lipids.

According to Hanahan (1960), phosphatidylethanolamine (a major component of cephalin) is found widely distributed in nature and next to lecithin is the most abundant naturally occurring phospholipid.

The findings with regard to cardiolipin and phosphatidyl inositol deserves further comment. Faure et al. (1950) isolated cardiolipin (polyglycerophosphatide) from ox liver and showed it to be similar to the cardiolipin isolated from beef heart. Macfarlane (1961) also demonstrated the presence of cardiolipin in ox liver and the percentage found by her (1 to 2 percent of the total phospholipid) is in agreement with the value of 1.4 percent of the total lipid reported by Rouser et al. (1963).

Hanahan et al. (1957) and Faure et al. (1950) reported the isolation of phosphatidyl inositol from beef liver and its presence in liver was confirmed in the studies of Rouser et al. (1963).

The presence of cardiolipin and phosphatidyl inositol (mono-phosphoinositide) in turkey tissues could not be ignored in this present study. Cardiolipin was probably the trace component in fraction I of the 3-solvent system which was then further resolved with small increments of methanol

(Figs. 9 through 13). Several workers reported that cardiolipin from tissues was usually eluted with 5 to 10 percent methanol in chloroform. The muscle phospholipids are characterized by a high content of cardiolipin (though not as high as that of heart muscle) and by high content of plasmalogens in comparison with other tissues (Davenport, 1964).

Oxygen uptake by total lipid and phospholipid fractions

Preliminary experiments on the rate of oxygen uptake by total lipid and various phospholipid fractions from turkey pectoralis major was done by use of the Warburg apparatus. Duplicate measurements of oxygen consumption were made on each fraction at 40°C. during a 54-hour period. Milliliters of oxygen uptake was plotted against time.

Packaged and unpackaged turkeys The results shown in Fig. 14 indicate that the oxygen consumption rate of total lipid from packaged and unpackaged turkey exhibit essentially the same pattern. Thus it appears that autoxidation of turkey lipids occurs slowly at freezer temperature even in the absence of an "evacuated" package. Although there was no appreciable difference in oxygen uptake during the induction period between samples, there was a slight increase in the volume of oxygen absorbed by fractions from packaged turkey after the rate became accelerated. This may be explained by the large initial sample size, 12.4 and 16.6

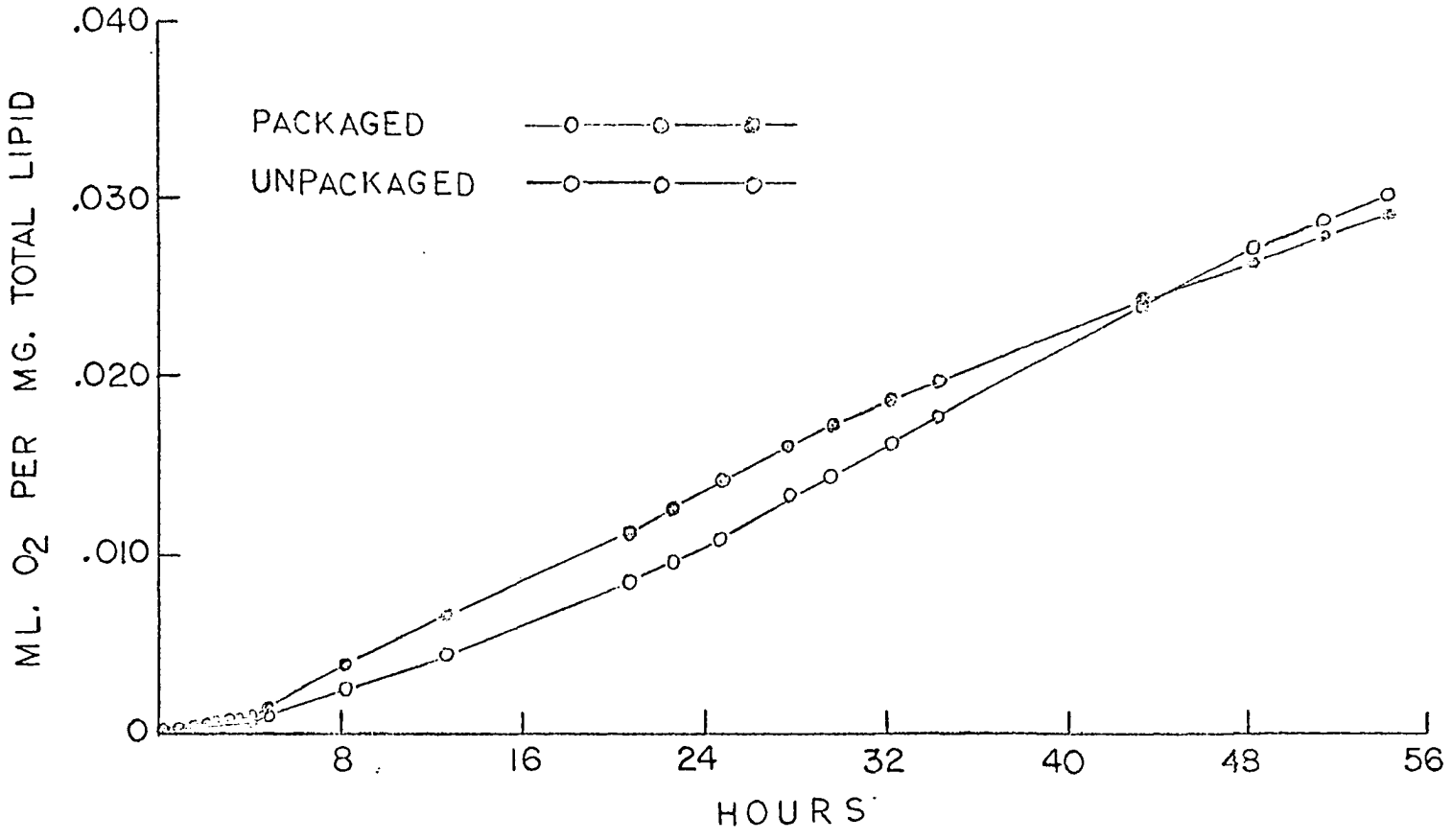


Fig. 14. Oxidation rate of total lipid from packaged and unpackaged turkey pectoralis major. Results from duplicate measurements are presented

mg compared to 9.0 and 10.0 mg from the unpackaged sample. Sufficient sample size is important to permit completion of the autoxidation induction period (Johnston and Frey, 1941). Those workers also observed in fatty oil samples that slight divergence in volume of oxygen absorbed after the rate becomes accelerated is typical.

Cooked and uncooked turkeys As might be expected, the relative rates of oxidation of total lipid, as well as the various phospholipid fractions, were found to vary between cooked and uncooked turkey. Oxygen uptake by total lipid from cooked and uncooked samples is shown in Fig. 15. The cooked samples had a shorter induction period. In addition, they consistently absorbed more oxygen than the uncooked samples. It has been established that TBA values increase rapidly following cooking of beef, veal, lamb and chicken (Timms and Watts, 1958). Zipser and Watts (1961) reported rapid oxidation of muscle lipids of mullet after cooking as shown by increases in TBA number and rancid odors. Younathan and Watts (1959) also found a marked difference in the rate of lipid oxidation in cured and uncured cooked meats.

Cephalin fraction The cephalin fraction (mixture of phosphatidylethanolamine, phosphatidylserine and their acetal and diester types) from the cooked turkey showed a shorter induction period and exhibited a faster rate of oxidation than was observed with uncooked turkey (Fig. 16). Mattsson

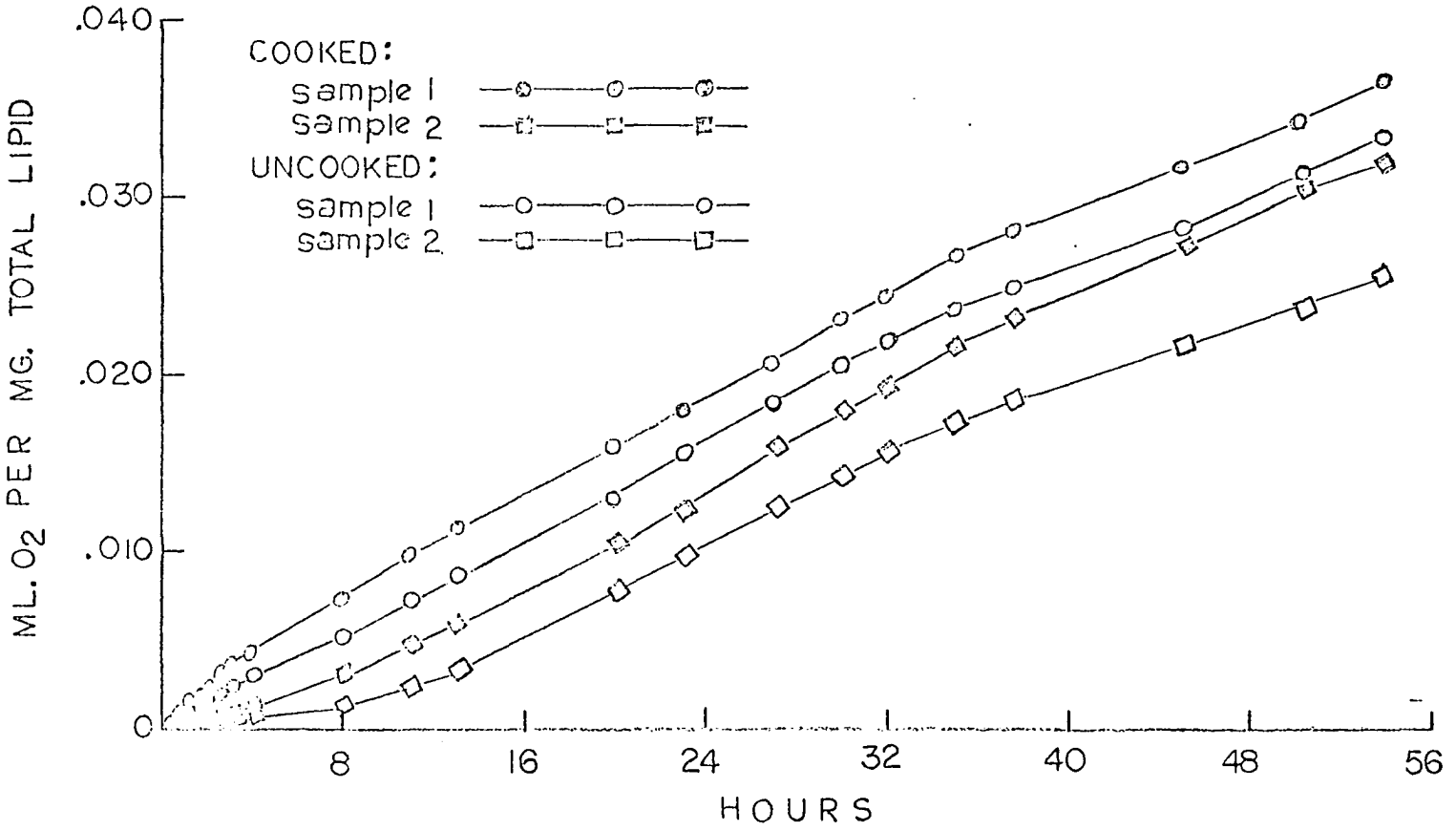
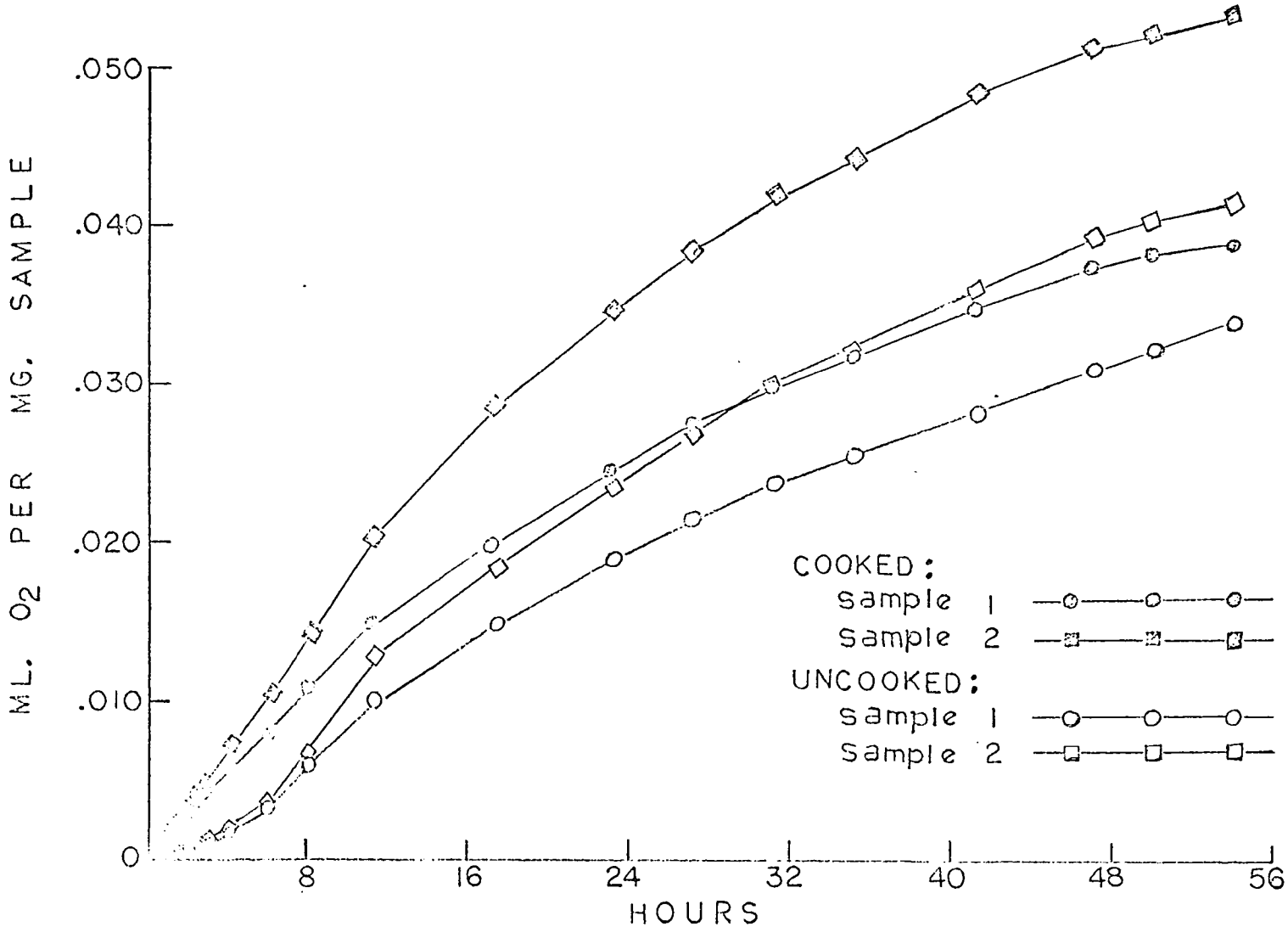


Fig. 15. Oxidation rate of total lipid from cooked and uncooked turkey pectoralis major. Results from duplicate measurements are presented

Fig. 16. Oxidation rate of cephalin fraction from cooked and uncooked turkey pectoralis major. Results from duplicate measurements are presented

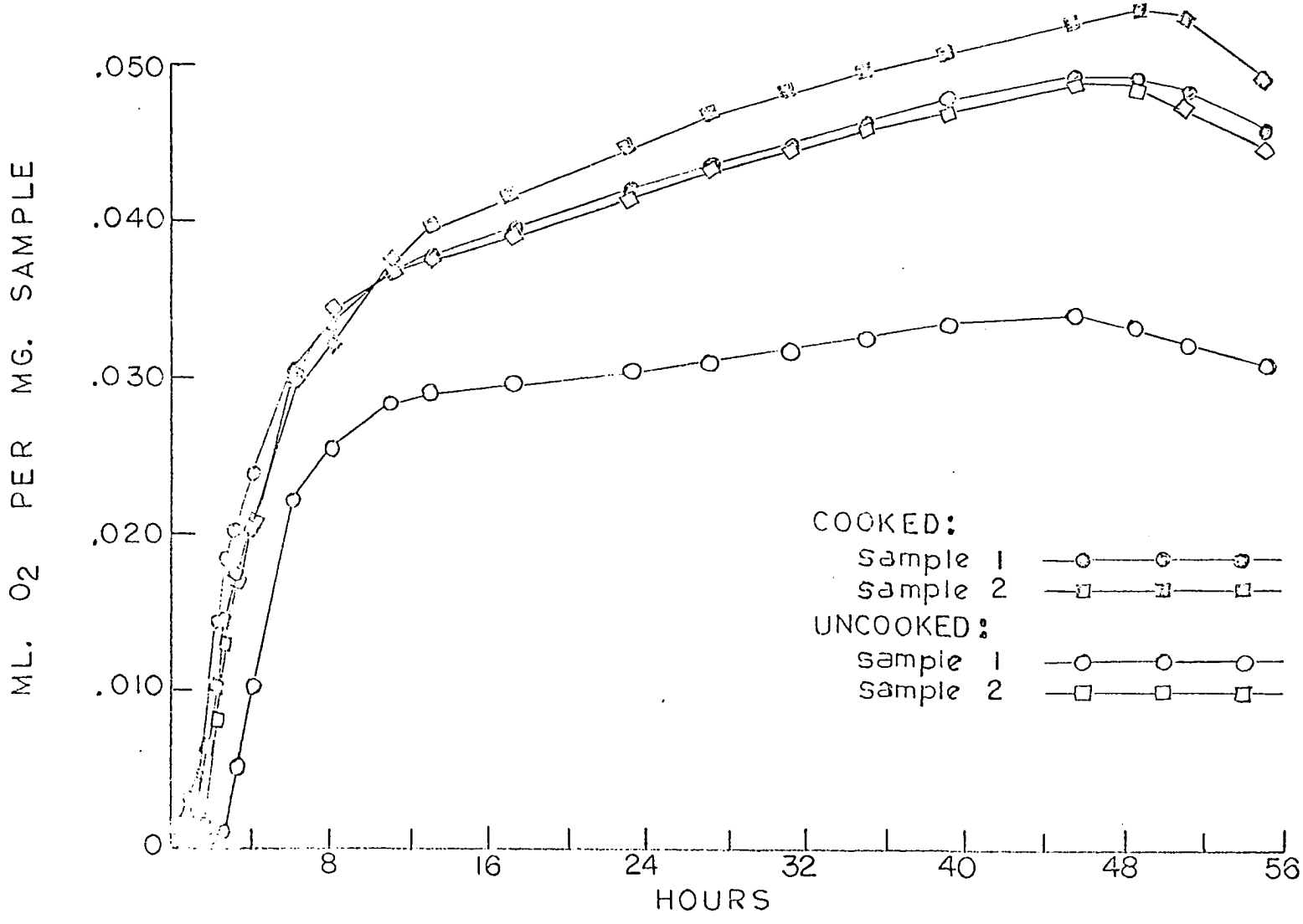

.050
.040
.030
.020
.010
0
ML. O2 PER MG. SAMPLE
8
16
24
32
40
48
56
HOURS
COOKED:
Sample 1
Sample 2
UNCOOKED:
Sample 1
Sample 2

and Swartling (1963) reported that rapid oxidation occurred with fractions containing ethanolamine and serine derivatives from milk. They found that a mixture of ethanolamine and serine derivatives belongs to the readily-oxidizable phospholipids. Of the individual phospholipids, phosphatidylserine was found to be most readily oxidized.

Lecithin fraction As shown in Fig. 17, the lecithin fraction oxidized most rapidly of the fractions studied. Also with this fraction, the induction period was longer and the oxygen absorbed less in uncooked turkey. Mattsson and Swartling (1963) reported that the lecithin fraction (phosphatidyl choline) reacted with oxygen fairly slowly. However, they observed that the difference between these two fractions (cephalin and lecithin) was found in the distribution of the serine derivatives which appeared in both fractions.

Sphingomyelin and non-phospholipid fractions The preliminary experiment indicated that these fractions were fairly resistant to oxygen uptake. Available samples that could be used were considered too small to measure any appreciable oxygen uptake. However, the data support that previously reported by Mattsson and Swartling (1963) who were unable to find any measurable amount of oxygen absorbed in a 50-hour period with the sphingomyelin fraction. The non-phospholipid fraction which consists mainly of triglycerides was also resistant to oxidation. These findings support

Fig. 17. Oxidation rate of lecithin fraction from cooked and uncooked turkey pectoralis major. Results from duplicate measurements are presented


.050
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ML. O2 PER MG. SAMPLE
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32
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HOURS
COOKED:
Sample 1
Sample 2
UNCOOKED:
Sample 1
Sample 2

earlier observations that the lipid fractions chiefly involved in oxidative changes are not the triglycerides but rather the proteolipids and phospholipids (Younathan and Watts, 1960).

Highly unsaturated fatty acids are quite widely distributed in small quantities as constituents of the fatty acid moiety of phospholipids (Klenk and Dreike, 1955). The concentration of unsaturated fatty acids in both meats and fish is highest in the phospholipid and proteolipid fractions (Lovern, 1956a and Reiser et al., 1960).

Although no definite conclusion can be drawn, the material does not contradict the existence of a relationship between the oxidation rate and the degree of unsaturation of the individual phospholipids in turkey tissues. This view was presented by Lea (1957), who studied thin-layer oxidation of egg phosphatidylethanolamine and phosphatidyl choline. Subfractions of phosphatidyl choline with high iodine values were found to oxidize faster than subfractions with lower iodine values. It would thus seem justified to regard the degree of unsaturation as one of the factors determining the rate of oxidation of phospholipids.

Lea (1957) found that the nature of the nitrogenous base of the phospholipid is also of importance to the oxidation rate. He reported that phosphatidylethanolamine oxidized much faster than phosphatidyl choline fractions of the same

iodine number. The degree of unsaturation of the phospholipids, the nature of its nitrogenous constituents and the electrolytic state of the polar groups are of fundamental importance for the oxidation rate (Mattsson and Swartling, 1963).

SUMMARY AND CONCLUSION

Total lipid and phospholipids were determined in turkey white and red muscles, liver, heart and gizzard.

Samples of lipids extracted from these tissues were separated into neutral lipids and phospholipids using silicic acid column chromatography. The phospholipids were further separated into major components. Phosphorous analyses were run on the phospholipid fractions to detect the portion containing phospholipids, and paper chromatography and infrared analyses were then run on the phospholipid fractions.

Neutral lipids were present but were not characterized further. All of the turkey tissues studied were found to contain the same 3 major phospholipid components: cephalin (phosphatidylethanolamine, phosphatidylserine and their acetal and diester types); lecithin (acetal and diester types) and sphingomyelin. Lysolecithin and/or mono-phosphoinositide may or may not be present in all the tissues, but trace amounts of it could be detected in most samples.

The decrease in phospholipid content of these turkey tissues with storage (0, 60 or 180 days, at -25°C) was statistically significant, whereas the total lipid content did not show any significant decrease.

Oxygen uptake measurements on total lipid and phospholipid fractions were done on samples from packaged and

unpackaged turkey, and cooked and uncooked turkey, by the use of the Warburg apparatus.

There was no appreciable difference in the oxygen uptake by samples from packaged and unpackaged turkey stored at freezer temperature for 17 days. This evidence suggests that autoxidation of turkey lipids occurs slowly at freezer temperature even in the absence of an "evacuated" package.

The rate of oxidation of total lipid, cephalin and lecithin were found to vary between cooked and uncooked turkey. The cooked samples had a shorter induction period and consistently absorbed more oxygen than samples from uncooked turkey.

The lecithin fraction oxidized most rapidly of the fractions studied. Sphingomyelin and non-phospholipid fractions were fairly resistant to oxygen uptake.

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APPENDIX

Table 8. Analyses of variance of percent phospholipid of dry tissue.

Sources of variation		d f	S S	M S	"F" ratio
Storage		2	32.575	16.287	21.375**
Replication	Within	3	6.354	2.118	
Birds within rep	storage	6	0.504	.084	
		9[a]	6.857	0.762	
Treatment		4	847.300	211.825	912.645**
S X T		8	57.337	7.167	30.879**
Residual		18	4.178	.232	
Total		41	948.248		

**Significant at P ≥ .01.

[a]Replications within storage, and birds within replication within storage represent, when combined, unaccounted for variation in the whole plot. A decision was made prior to the experiment to use the combined mean squares to test "storage", assuming these individually would approximate a 1:1 ratio. However, there was a surprisingly large ratio between the two, which cannot be explained.

Table 9. Analyses of variance of percent phospholipid of total lipid

Sources of variation		d f	S S		M S		"F" ratio
Storage		2	2,239.915		1,119.958		4.357*
Replication	Within	3	1,632.477	2,313.240	544.159	257.027	
Birds within rep	storage	6	680.763		113.461		
		9					
Treatment		4	5,926.715		1,481.679		37.598**
S X T		8	744.238		93.030		2.360
Residual		18	748.742		39.408		
Total		41	11,972.850				

*Significant at P ≥ .05.
**Significant at P ≥ .01.

Table 10. Analyses of variance of percent total lipid of dry tissue

Sources of variation		d f	S S	M S	"F" ratio
Storage		2	3.374	1.687	1.654
Replication	Within	3	2.027	.676	
Birds within rep	storage	6	7.155	1.193	
		9	9.182	1.020	
Treatment		4	992.407	248.102	338.493**
S X T		8	36.507	4.564	6.245**
Residual		18	13.154	0.731	
Total		41	1,054.625		

**Significant at P≥.01.